(54) Title: OSTEOGENIC DEVICES

(57) Abstract

Disclosed are 1) amino acid sequence data, structural features, and various other data characterizing a human osteogenic protein, OP1, 2) osteogenic devices comprising a heat treated xenogenic bone collagen matrix containing osteogenic protein, 3) methods of producing osteogenic proteins using recombinant DNA technology and 4) use of osteogenic devices to mimic the natural course of endochondral bone formation in mammals.

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## OSTEOGENIC DEVICES

### Reference to Related Applications

This application is a continuation-in-part
of copending U.S. application Serial No. 422,699
filed October 17, 1989 entitled "Osteogenic Devices,"
and U.S. application Serial No. 483,913, filed
February 22, 1990, entitled "Bone Collagen Matrix for
Implants."

### Background of the Invention

This invention relates to osteogenic
devices, to genes encoding proteins which can induce
new bone formation in mammals, and to methods for the
production of these proteins in mammalian cells using
recombinant DNA techniques.  The invention also
relates to matrix materials useful for allogenic or
xenogenic implants and which act as a carrier of the
osteogenic protein to induce new bone formation in
mammals, and to bone and cartilage repair procedures
using the osteogenic devices.

Mammalian bone tissue is known to contain
one or more proteinaceous materials, presumably
active during growth and natural bone healing, which

can induce a developmental cascade of cellular events resulting in endochondral bone formation. This active factor (or factors) has variously been referred to in the literature as bone morphogenetic or morphogenic protein, bone inductive protein, osteogenic protein, osteogenin, or osteoinductive protein.

The developmental cascade of bone differentiation consists of recruitment of mesenchymal cells, proliferation of progenitor cells, calcification of cartilage, vascular invasion, bone formation, remodeling, and finally marrow differentiation (Reddi (1981) Collagen Rel. Res. 1:209-226).

Though the precise mechanisms underlying these phenotypic transformations are unclear, it has been shown that the natural endochondral bone differentiation activity of bone matrix can be dissociatively extracted and reconstituted with inactive residual collagenous matrix to restore full bone induction activity (Sampath and Reddi (1981) Proc. Natl. Acad. Sci. USA 78:7599-7603). This provides an experimental method for assaying protein extracts for their ability to induce endochondral bone in vivo. Several species of mammals produce closely related protein as demonstrated by cross species implant experiments (Sampath and Reddi (1983) Proc. Natl. Acad. Sci. USA 80:6591-6595).

The potential utility of these proteins has been recognized widely. It is contemplated that the availability of the protein would revolutionize

orthopedic medicine, certain types of plastic
surgery, and various periodontal and craniofacial
reconstructive procedures.

The observed properties of these protein
fractions have induced an intense research effort in
several laboratories directed to isolating and
identifying the pure factor or factors responsible
for osteogenic activity. The current state of the
art of purification of osteogenic protein from
mammalian bone is disclosed by Sampath et al. (1987)
Proc. Natl. Acad. Sci. USA 80. Urist et al. (1984)
Proc. Soc. Exp. Biol. Med. 173:194-199 disclose a
human osteogenic protein fraction which was extracted
from demineralized cortical bone by means of a
calcium chloride-urea inorganic-organic solvent
mixture, and retrieved by differential precipitation
in guanidine-hydrochloride and preparative gel
electrophoresis. The authors report that the protein
fraction has an amino acid composition of an acidic
polypeptide and a molecular weight in a range of
17-18 kD.

Urist et al. (1984) Proc. Natl. Acad. Sci.
USA 81:371-375 disclose a bovine bone morphogenetic
protein extract having the properties of an acidic
polypeptide and a molecular weight of approximately
18 kD. The authors reported that the protein was
present in a fraction separated by hydroxyapatite
chromatography, and that it induced bone formation in
mouse hindquarter muscle and bone regeneration in
trephine defects in rat and dog skulls. Their method
of obtaining the extract from bone results in
ill-defined and impure preparations.

European Patent Application Serial No.
148,155, published October 7, 1985, purports to
disclose osteogenic proteins derived from bovine,
porcine, and human origin.  One of the proteins,
designated by the inventors as a P3 protein having a
molecular weight of 22-24 kD, is said to have been
purified to an essentially homogeneous state.  This
material is reported to induce bone formation when
implanted into animals.

International Application No. PCT/O87/01537,
published January 14, 1988 (Int. Pub. No.
WO88/00205), discloses an impure fraction from bovine
bone which has bone induction qualities.  The named
applicants also disclose putative "bone inductive
factors" produced by recombinant DNA techniques.
Four DNA sequences were retrieved from human or
bovine genomic or cDNA libraries and expressed in
recombinant host cells.  While the applicants stated
that the expressed proteins may be bone morphogenic
proteins, bone induction was not demonstrated,
suggesting that the recombinant proteins are not
osteogenic.  The same group reported subsequently
(Science, 242:1528, Dec. 1988) that three of the
four factors induce cartilage formation, and
postulate that bone formation activity "is due to a
mixture of regulatory molecules" and that "bone
formation is most likely controlled ... by the
interaction of these molecules."  Again, no bone
induction was attributed to the products of
expression of the cDNAs.  See also Urist et al., EPO
212,474 entitled Bone Morphogenic Agents.

        Wang et al. (1988) Proc. Nat. Acad. Sci. USA
85: 9484-9488, disclose the purification of a bovine
bone morphogenetic protein from guanidine extracts of
demineralized bone having cartilage and bone
formation activity as a basic protein corresponding
to a molecular weight of 30 kD determined from gel
elution.  Purification of the protein yielded
proteins of 30, 18 and 16 kD which, upon separation,
were inactive.  In view of this result, the authors
acknowledged that the exact identity of the active
material had not been determined.

        Wang et al. (1990) Proc. Nat. Acad. Sci. USA
87: 2220-2227 describes the expression and partial
purification of one of the cDNA sequences described
in PCT 87/01537.  Consistent cartilage and/or bone
formation with their protein requires a minimum of
600 ng of 50% pure material.

        International Application No. PCT/89/04458
published April 19, 1990 (Int. Pub. No. WO90/003733),
describes the purification and analysis of a family
of osteogenic factors called "P3 OF 31-34".  The
protein family contains at least four proteins, which
are characterized by peptide fragment sequences.  The
impure mixture P3 OF 31-34 is assayed for osteogenic
activity.  The activity of the individual proteins is
neither assessed nor discussed.

        It has been found that successful
implantation of the osteogenic factors requires
association of the proteins with a suitable carrier
material capable of maintaining the proteins at an in
vivo site of application.  The carrier should be

biocompatible, biodegradable and porous enough to
allow cell infiltration.  The insoluble collagen
particles remaining after guanidine extraction and
delipidation of pulverized bone generally have been
found effective in allogenic implants in some
species.  However, studies have shown that while
osteoinductive proteins are useful cross species, the
collagenous bone matrix generally used for inducing
endochondral bone formation is species specific
(Sampath and Reddi (1983) Proc. Nat. Acad. Sci. USA
80:6591-6594).  Demineralized, delipidated, extracted
xenogenic bone matrix carriers implanted in vivo
invariably fail to induce osteogenesis, presumably
due to inhibitory or immunogenic components in the
bone matrix.  Even the use of allogenic bone matrix
in osteogenic devices may not be sufficient for
osteoinductive bone formation in many species.  For
example, allogenic, subcutaneous implants of
demineralized, delipidated monkey bone matrix is
reported not to induce bone formation in the monkey.
(Asperberg et al. (1988) J. Bone Joint Surg. (Br)
70-B:625-627).

        U.S. 4,563,350, published January 7, 1986,
discloses the use of trypsinized bovine bone matrix
as a xenogenic matrix to effect osteogenic activity
when implanted with extracted, partially purified
bone-inducing protein preparations.  Bone formation
is said to require the presence of at least 5%, and
preferably at least 10%, non-fibrillar collagen.  The
authors claim that removal of telopeptides which are
responsible in part for the immunogenicity of
collagen preparations is more suitable for xenogenic
implants.

European Patent Application Serial No.
309,241, published 3/29/89, discloses a device for
inducing endochondral bone formation comprising an
osteogenic protein preparation, and a matrix carrier
comprising 60-98% of either mineral component or bone
collagen powder and 2-40% atelopeptide
hypoimmunogenic collagen.

Deatherage et al. (1987) Collagen Rel. Res.
7:2225-2231, purport to disclose an apparently
xenogenic implantable device comprising a bovine bone
matrix extract that has been minimally purified by a
one-step ion exchange column and reconstituted,
highly purified human Type-I placental collagen.

U.S. 3,394,370, published 7/19/83, describes
a matrix of reconstituted collagen purportedly useful
in xenogenic implants. The collagen fibers are
treated enzymatically to remove potentially
immunogenic telopeptides (also the primary source of
interfibril crosslinks) and are dissolved to remove
associated non-collagen components. The matrix is
formulated by dispersing the reconstituted collagen
in acetic acid to form a disordered matrix of
elementary collagen molecules that is then mixed with
osteogenic factor and lyophilized to form a
"semi-rigid foam or sponge" that is preferably
crosslinked. The formulated matrix is not tested in
vivo.

U.S. 4,172,128, published 10/23/79,
describes a method for degrading and regenerating
bone-like material of reduced immunogenicity, said to

be useful cross-species. Demineralized bone
particles are treated with a swelling agent to
dissolve any associated mucopolysaccharides
(glycosaminoglycans) and the collagen fibers
subsequently dissolved to form a homogenous colloidal
solution. A gel of reconstituted fibers then can be
formed using physiologically inert
mucopolysaccharides and an electrolyte to aid in
fibril formation.

It is an object of this invention to provide
osteogenic devices comprising matrices containing
dispersed osteogenic protein produced from
recombinant DNA and capable of bone induction in
allogenic and xenogenic implants. Another object is
to provide recombinant osteogenic proteins expressed
from mammalian cells and capable of inducing
endochondral bone formation in mammals, including
humans. Still another object is to provide genes
encoding osteogenic proteins and methods for their
production using recombinant DNA techniques. Yet
another object is to provide a biocompatible, in vivo
biodegradable matrix capable, in combination with an
osteoinductive protein, of producing endochondral
bone formation in mammals, including humans.

These and other objects and features of the
invention will be apparent from the description,
drawings, and claims which follow.

## Summary of the Invention

This invention provides osteogenic proteins and devices which, when implanted in a mammalian body, can induce at the locus of the implant the full developmental cascade of endochondral bone formation including vascularization, mineralization, and bone marrow differentiation. The devices comprise a carrier material, referred to herein as a matrix, having the characteristics disclosed below, and containing dispersed osteogenic protein produced using recombinant DNA techniques and expressed from eukaryotic cells, preferably mammalian cells.

Preferred embodiments of the recombinant protein dispersed in the matrix disclosed herein closely mimic the physiological activity of native form protein extracted from natural sources and reconstituted in allogenic demineralized bone powder matrix material. The preferred proteins have a specific activity far higher than any biosynthetic material heretofore reported, an activity which, within the limits of precision of the activity assay, appears essentially identical to the substantially pure material produced as set forth in copending application Serial No. 179,406 filed April 8, 1988 (PCT US/89 01453). Thus, this application discloses how to make and use osteogenic devices which induce the full developmental cascade of endochondral bone formation essentially as it occurs in natural bone healing.

A key to these developments was the elucidation of amino acid sequence and structure data

of native osteogenic protein. A protocol was developed which results in retrieval of active, substantially pure osteogenic protein from mammalian bone having a half-maximum bone forming activity of about 0.8 to 1.0 ng per mg of implant. The availability of the material enabled the inventors to elucidate all structural details of the protein necessary to achieve bone formation. Knowledge of the protein's amino acid sequence and other structural features enabled the identification and cloning of native genes.

Consensus DNA sequences based on partial sequence data and observed homologies with regulatory proteins disclosed in the literature were used as probes for extracting genes encoding osteogenic protein from genomic and cDNA libraries. One of the consensus sequence probes isolated a previously unidentified DNA sequence, portions of which, when ligated, encoded a protein comprising a region capable of inducing endochondral bone formation when properly modified, incorporated in a suitable matrix, and implanted as disclosed herein. The protein, referred to herein as OP1, as well as various truncated forms and fusion constructs, has been expressed in E. coli and various mammalian cells from the full length cDNA sequence and various truncated synthetic DNAs, and has been discovered to exhibit osteogenic activity as a homodimer or as a heterodimer with BMP2, another osteogenic protein extracted from human DNA libraries with the consensus sequence probes.

Characterization of the OP1 gene and
identification of the DNA and amino acid sequence
necessary for activity has allowed expression of the
gene in mammalian cells.  Mammalian cell expression
of recombinant proteins, particularly mammalian
proteins intended for therapeutic use, is generally
thought to yield proteins having a structure most
like that of the natural material.  This is
particularly true for secreted proteins which require
particular post-translational modifications, such as
glycosylation, which are not carried out in
procaryotic systems.  While expression of the OP1
gene in E. coli has shown that the unglycosylated
form of the protein has osteogenic activity, there
may be other as yet undetermined functions for the
oligosaccharides, relating to protein stability,
solubility, or immunogenicity, for example.  In
addition, purification of proteins secreted into
culture media provide an alternative to extraction of
induced proteins from procaryotic inclusion bodies.

Mammalian cell expression of the gene also
has allowed determination of the N-terminus of the
mature protein.  The amino acid sequence of what is
believed to be the mature form of OP1 is (Seq. ID No.
1):

<u>OP1-18</u>

```
1                              10
S   T   G   S   K   Q   R   S   Q   N   R   S   K   T   P
                20                                      30
K   N   Q   E   A   L   R   M   A   N   V   A   E   N   S
                                    40
S   S   D   Q   R   Q   A   C   K   K   H   E   L   Y   V
                50                                      60
S   F   R   D   L   G   W   Q   D   W   I   I   A   P   E
                                    70
G   Y   A   A   Y   Y   C   E   G   E   C   A   F   P   L
                80                                      90
N   S   Y   M   N   A   T   N   H   A   I   V   Q   T   L
                                    100
V   H   F   I   N   P   E   T   V   P   K   P   C   C   A
                110                                     120
P   T   Q   L   N   A   I   S   V   L   Y   F   D   D   S
                                    130
S   N   V   I   L   K   K   Y   R   N   M   V   V   R   A

C   G   C   H.
```

Recombinantly-produced OP1 also is active in several forms truncated at the protein's N-terminus. One main species of truncated OP1 is (Seq. ID No. 2):

<u>OP1-16S</u>

```
                        8   10
                        S   Q   N   R   S   K   T   P
                20                                      30
    K   N   Q   E   A   L   R   M   A   N   V   A   E   N   S
                                        40
    S   S   D   Q   R   Q   A   C   K   K   H   E   L   Y   V
                    50                                      60
    S   F   R   D   L   G   W   Q   D   W   I   I   A   P   E
                                        70
    G   Y   A   A   Y   C   E   G   E   C   A   F   P   L
                    80                                      90
    N   S   Y   M   N   A   T   N   H   A   I   V   Q   T   L
                                        100
    V   H   F   I   N   P   E   T   V   P   K   P   C   C   A
                    110                                     120
    P   T   Q   L   N   A   I   S   V   L   Y   F   D   D   S
                                        130
    S   N   V   I   L   K   K   Y   R   N   M   V   V   R   A

    C   G   C   H.
```

Four other active shorter OP1 sequences
are:

<u>OP1-16L</u> (Seq. ID No. 3)

```
               21                              30
               L   R   M   A   N   V   A   E   N   S
                                40
   S   S   D   Q   R   Q   A   C   K   K   H   E   L   Y   V
                50                                      60
   S   F   R   D   L   G   W   Q   D   W   I   I   A   P   E
                                70
   G   Y   A   A   Y   Y   C   E   G   E   C   A   F   P   L
                80                                      90
   N   S   Y   M   N   A   T   N   H   A   I   V   Q   T   L
                               100
   V   H   F   I   N   P   E   T   V   P   K   P   C   C   A
               110                                     120
   P   T   Q   L   N   A   I   S   V   L   Y   F   D   D   S
                               130
   S   N   V   I   L   K   K   Y   R   N   M   V   V   R   A

   C   G   C   H ;
```

<u>OP1-16M</u> (Seq. ID No. 4)

```
               23                              30
               M   A   N   V   A   E   N   S
                                40
   S   S   D   Q   R   Q   A   C   K   K   H   E   L   Y   V
                50                                      60
   S   F   R   D   L   G   W   Q   D   W   I   I   A   P   E
                                70
   G   Y   A   A   Y   Y   C   E   G   E   C   A   F   P   L
                80                                      90
   N   S   Y   M   N   A   T   N   H   A   I   V   Q   T   L
                               100
   V   H   F   I   N   P   E   T   V   P   K   P   C   C   A
               110                                     120
   P   T   Q   L   N   A   I   S   V   L   Y   F   D   D   S
                               130
   S   N   V   I   L   K   K   Y   R   N   M   V   V   R   A

   C   G   C   H ;
```

## OP1-16A (Seq. ID. No. 5)

```
                                     24                        30
                                      A   N   V   A   E   N   S
                                         40
      S   S   D   Q   R   Q   A   C   K   K   H   E   L   Y   V
                     50                                      60
      S   F   R   D   L   G   W   Q   D   W   I   I   A   P   E
                                         70
      G   Y   A   A   Y   Y   C   E   G   E   C   A   F   P   L
                     80                                      90
      N   S   Y   M   N   A   T   N   H   A   I   V   Q   T   L
                                        100
      V   H   F   I   N   P   E   T   V   P   K   P   C   C   A
                    110                                     120
      P   T   Q   L   N   A   I   S   V   L   Y   F   D   D   S
                                        130
      S   N   V   I   L   K   K   Y   R   N   M   V   V   R   A

      C   G   C   H ; and
```

## OP1-16V (Seq. ID. No. 6)

```
                                                             30
                                          V   A   E   N   S
                                         40
      S   S   D   Q   R   Q   A   C   K   K   H   E   L   Y   V
                     50                                      60
      S   F   R   D   L   G   W   Q   D   W   I   I   A   P   E
                                         70
      G   Y   A   A   Y   Y   C   E   G   E   C   A   F   P   L
                     80                                      90
      N   S   Y   M   N   A   T   N   H   A   I   V   Q   T   L
                                        100
      V   H   F   I   N   P   E   T   V   P   K   P   C   C   A
                    110                                     120
      P   T   Q   L   N   A   I   S   V   L   Y   F   D   D   S
                                        130
      S   N   V   I   L   K   K   Y   R   N   M   V   V   R   A

      C   G   C   H.
```

These 6 species of OP1 have been tested for
osteogenic activity in vivo and all have been shown
to induce endochondral bone formation in a
dose-dependent manner when implanted in a mammal in
association with a suitable matrix.  The specific
activity of these species is close to that of the
substantially pure, naturally-sourced osteogenic
protein.  Moreover, these proteins mimic the activity
of the naturally-sourced material more closely than
other osteogenic protein preparations heretofore
reported.

Recombinantly produced OP1 is expressed as a
glycosylated homodimer in mammalian cells.
Homodimers of OP1-18 have an apparent molecular
weight of about 36 kD, when oxidized, and about 18 kD
when reduced, as determined by SDS-PAGE gels.
OP1-16S, OP1-16V, OP1-16M, OP1-16A and OP1-16L have
an apparent molecular weight of about 16kD, when
reduced, and homodimers of these proteins, as well as
heterodimers with OP1-18 have an apparent molecular
weight within the range of about 30-36 kD when
oxidized, as determined by SDS-PAGE gels.  In the
reduced state, these proteins have no detectable
osteogenic activity.

OP1 now has been expressed in a number of
different mammalian cells, all of which glycosylate
and process the protein after translation.  While the
precise structure of the oligosaccharide side chains
may vary among the different cell lines, in all cases
the expressed sequence is osteogenically active in a
specific and dose dependent manner.

The invention is not limited to those specific constructs. Thus, the osteogenic proteins of this invention may include forms having varying glycosylation patterns, varying N-termini, a family of related proteins having regions of amino acid sequence homology, and active truncated or mutated forms of the native amino acid sequence, produced by expression of recombinant DNA in eucaryotic host cells. Active sequences useful in an osteogenic device of this invention is envisioned to include osteogenic proteins having at least a 70% sequence homology, preferably at least 80%, with the amino acid sequence of OP1-16V. This includes longer forms of the protein, as well as allelic variants and muteins.

Thus, in view of this disclosure, skilled genetic engineers can isolate genes from cDNA or genomic libraries which encode appropriate amino acid sequences, or construct DNAs from oligonucleotides, and then can express them in various types of eucaryotic host cells to produce large quantities of active proteins capable of inducing bone formation in mammals, including humans.

The osteogenic proteins are useful in clinical applications in conjunction with a suitable delivery or support system (matrix). The matrix comprises biocompatible, protein-extracted, mineral-free, delipidated, insoluble Type-I bone collagen particles which may be allogenic or xenogenic to the host. The particles preferably are treated with a fibril-modifying agent such as hot water or other fibril-modifying solvents, to alter

the particle morphology, i.e., to increase the
intraparticle porosity and the surface area of the
particles. The particles are packed together to form
the matrix. The spaces among the particles must be
of a dimension to permit progenitor cell migration
and subsequent cell differentiation and
proliferation. The particle size should be within
the range of 70 - 850 μm, preferably 150μm - 420μm.
The matrix may be fabricated by close packing the
particles into a shape spanning the bone defect, or
by otherwise shaping the packed particles as
desired. The matrix is biocompatible
(non-inflammatory) and biodegradable in vivo, and
serves as a "temporary scaffold" and substratum for
recruitment of migratory progenitor cells, and as a
base for their subsequent anchoring and
proliferation. As disclosed herein, the matrix may
be combined with osteogenic protein to induce
endochondral bone formation reliably and reproducibly
in a mammalian body.

The development of this matrix material
resulted from the discovery of key features required
for successful implantation of xenogenic bone matrix
and osteogenic protein. Studies indicated that
osteogenic devices comprising substantially pure
osteogenic protein and allogenic demineralized,
delipidated protein-extracted bone matrices must have
interstices dimensioned to permit the influx,
proliferation and differentiation of migratory
progenitor cells. It was also observed that
osteogenic devices comprising xenogenic bone matrices
induce little or no endochondral bone formation in
vivo. The absence of bone formation by xenogenic

matrices generally has been thought to be due to an immunogenic or inhibitory response to components still present in the matrix (e.g., the collagen telopeptides or associated non-collagenous glycoproteins.)

It has now been discovered that the overall specific particle surface area (surface area/unit mass), the degree of porosity and micropitting, and the size of the micropits and pores of the matrix particles is important for successful xenogenic implants, and even for allogenic implants of certain species.

Panels A and B of FIGURE 1 are scanning electron micrographs showing the particle structure of demineralized, guanidine-extracted bone matrix from rat and calf, respectively. As can be seen from the SEMs, there is a significantly greater inherent porosity, or surface area, in rat bone matrix than in bovine bone matrix. It has been discovered that increasing the porosity and intraparticle surface area of bone matrix can promote osteogenic induction as evidenced by rat collagenous bone matrix implants. This is achieved by treating collagenous bone matrix with certain solvents or heat so as to alter its morphology. Agents suitable for this purpose are disclosed herein and are termed collagen fibril-modifying agents.

Thus, one aspect of this invention includes osteogenic devices comprising matrices which have been treated to increase the surface area and porosity of matrix collagen particles substantially.

The currently preferred fibril-modifying
agent useful in the osteogenic devices of this
invention is a heated aqueous medium, most preferably
water. Heating demineralized delipidated guanidine
extracted bone collagen in water at high temperature
(37°-65°, preferably 45°-60°C) for approximately one
hour is generally sufficient to achieve the desired
surface morphology. Although the mechanism is not
clear, it is hypothesized that the heat treatment
alters the collagen fibrils, resulting in an increase
in the particle's surface area. Thus, bone matrix
may be treated at various elevated temperatures in
water (1g/30ml) with stirring and then filtered.
Treatment of insoluble collagen in water by
increasing temperature results initially in a melting
transition (Tm), the temperature required to go from
one-quarter to three quarters of the total transition
from helical structure to non-helical. Thereafter
the fibrils will shrink abruptly a fraction of length
at some higher temperature, designated as the
shrinkage temperature (Ts). Ts is normally higher
than Tm, reflecting the added stability contributed
by molecular packing. At pHs below approximately 5,
both the Tm and Ts values decrease for heated
collagen.

Examination of solvent treated bone
collagenous matrix shows that demineralized
guanidine-extracted xenogenic bovine bone comprises a
mixture of additional materials and that extracting
these materials can improve matrix properties.
Chromatographic separation of components in the
extract, followed by addition back to active matrix
of the various extract fractions corresponding to the

chromatogram peaks, indicates that there is a
fraction which can inhibit the osteoinductive
effect. The identity of the substance or substances
in this inhibiting fraction has not as yet been
determined. In one aspect of this invention, a
matrix is provided comprising Type-I bone collagen
particles of the type described above, further
characterized in that they are depleted in this
inhibiting component.

In view of this disclosure, one skilled in
the art can create a biocompatible matrix of choice
having a desired porosity or surface microtexture
useful in the production of osteogenic devices, and
useful in other implantable contexts, e.g., as a
packing to promote bone induction, or as a
biodegradable sustained release implant.

The osteogenic proteins and implantable
osteogenic devices disclosed herein will permit the
physician to obtain optimal predictable bone
formation to correct, for example, acquired and
congenital craniofacial and other skeletal or dental
anomalies (Glowacki et al. (1981) Lancet 1:959-963).
The devices may be used to induce local endochondral
bone formation in non-union fractures as demonstrated
in animal tests, and in other clinical applications
including periodontal applications where bone
formation is required. Another potential clinical
application is in cartilage repair, for example, in
the treatment of osteoarthritis.

Brief Description of the Drawing

        The foregoing and other objects of the
invention, the various features thereof, as well as
the invention itself, may be more fully understood
from the following description, when read together
with the accompanying drawings, in which:

        FIGURE 1A and 1B are scanning electron
micrographs (5000X) of demineralized, delipidated (A)
rat bone collagen particles, and (B) bovine bone
collagen particles;

        FIGURE 2-1 and 2-2 represent the full length
cDNA and encoded amino acid sequence of the prepro
form of human OP1 protein (Seq. ID No. 7);

        FIGURE 3A through 3F are restriction maps of
various expression vectors designed for the mammalian
cell expression of OP1;

        FIGURE 4 is a photoreproduction of western
blots (immunoblots) comparing OP1 expressed from:
COS cells - (A) pH717, (B) pH731; CHO cells - (C)
pH754, (D) pH752; and BSC cells - (E) pH717, (F)
pW24;

        FIGURE 5A-C are (1) elution profiles and (2)
photoreproductions of SDS-PAGE gels expressed from
BSC cells and purified (in order) on:  (A)
S-Sepharose, (B) phenyl-Sepharose, and (c) C-18
columns;

FIGURE 6 is a photoreproduction of SDS-PAGE gels of OP1 purified from BSC cells, comparing the intact dimer under oxidized conditions (36 kD, lane 1) and the corresponding monomer, after reduction with dithiothreitol (18kD, lane 5), with molecular weight standards (lanes 2-4);

FIGURE 7A through 7D are scanning electron micrographs (approx. 1000X) of demineralized, delipidated bovine bone matrix heat treated in water at (A) 37° C, (B) 45° C, (C) 55° C, and (D) 65° C;

FIGURE 8 is a 214 nm absorbance tracing of the extract isolated from hot water-treated bovine matrix, identifying the inhibitory effect of individual fractions on in vivo bone formation;

FIGURE 9A and 9B are bar graphs showing the inhibitory effect of hot water-treated matrix extract on OP1 activity, as measured by (A) alkaline phosphatase activity and (B) calcium content in day 12 implants, vs. increasing concentration of extract solvent;

FIGURE 10A-F are photomicrographs (220x) of allogenic implants of OP1 expressed from COS, BSC and CHO cells, and which follow the developmental cascade of endochondral bone osteogenesis;

FIGURE 11 is a photomicrograph showing the histology (day 12) of a xenogenic implant of this invention using OP1 expressed from BSC cells and hot water-treated xenogenic bovine matrix;

FIGURE 12 describes the dose dependence of osteogenic implants for day 12 implants, as determined by alkaline phosphatase activity and calcium content, for allogenic implants containing OP1 expressed from COS, BSC and CHO cells; and

FIGURE 13A and 13B are bar graphs showing the dose dependence of OP1 expressed in COS and BSC cells, as measured by (A) alkaline phosphatase activity and (B) calcium content in xenogenic implants (day 12), vs increasing concentration of protein (dose curve in ng).

Description

        Purification protocols first were developed
which enabled isolation of the osteogenic protein
present in crude protein extracts from mammalian
bone.  (See PCT US 89/01453, and U.S. Serial No.
179,406 filed April 8, 1988).  The development of the
procedure, coupled with the availability of fresh
calf bone, enabled isolation of substantially pure
bovine osteogenic protein (BOP).  BOP was
characterized significantly; its ability to induce
cartilage and ultimately endochondral bone growth in
cat, rabbit, and rat were demonstrated and studied;
it was shown to be able to induce the full
developmental cascade of bone formation previously
ascribed to unknown protein or proteins in
heterogeneous bone extracts.  This dose dependent and
highly specific activity was present whether or not
the protein was glycosylated (see U.S. Serial
No. 232,630 filed 8/15/88 and Sampath et al., (1990)
J. Biol. Chem. 265: pp. 13198-13205).  Sequence data
obtained from the bovine materials suggested probe
designs which were used to isolate human genes.  The
OP human counterpart proteins have now been expressed
and extensively characterized.

        These discoveries enabled preparation of
DNAs encoding totally novel, non-native protein
constructs which individually as homodimers and
combined with other species as heterodimers are
capable of producing true endochondral bone (see PCT
89/01469, filed 4/7/89 and US Serial No. 315,342,
filed 2/23/89).  They also permitted expression of
the natural material, truncated forms, muteins,

analogs, fusion proteins, and various other variants and constructs, from cDNAs and genomic DNAs retrieved from natural sources or from synthetic DNA produced using the techniques disclosed herein and using automated, commercially available equipment. The DNAs may be expressed using well established molecular biology and recombinant DNA techniques in procaryotic or eucaryotic host cells, and may be oxidized and refolded in vitro if necessary, to produce biologically active protein.

One of the DNA sequences isolated from genomic and cDNA libraries encoded a previously unidentified gene, referred to herein as OP1. The protein encoded by the isolated DNA was identified originally by amino acid homology with proteins in the TGF-ß family. Consensus splice signals were found where amino acid homologies ended, designating exon-intron boundaries. Three exons were combined to obtain a functional TGF-ß-like domain containing seven cysteines. (See, for example, U.S. Serial No. 315,342 filed 2/23/80, or Ozkaynak, E. et al., (1990) EMBO. 9: pp. 2085-2093).

The full-length cDNA sequence for OP1, including the amino acid sequence it encodes, is represented in Figure 2. This full length cDNA sequence of OP1, as well as various truncated forms of the gene, and fused genes, have been expressed in E. coli and shown to have osteogenic activity when implanted in a mammal in association with a matrix.

The native form protein is expressed originally in a "prepro" form which includes a signal

peptide sequence for appropriate secretion of the
protein.  The signal peptide cleavage site is
underlined in Figure 2.  Removal of the signal
peptide yields the "pro" form of the protein, which
is processed upon secretion to yield the mature
sequence.  The cleavage site yielding the mature
sequence is indicated by an arrow in Figure 2.  The
amino acid sequence of what is believed to be the
mature form is (Seq. ID No. 1):


<u>OP1-18</u>


```
1                                    10
S   T   G   S   K   Q   R   S   Q   N   R   S   K   T   P
                20                              30
K   N   Q   E   A   L   R   M   A   N   V   A   E   N   S
                            40
S   S   D   Q   R   Q   A   C   K   K   H   E   L   Y   V
                50                              60
S   F   R   D   L   G   W   Q   D   W   I   I   A   P   E
                            70
G   Y   A   A   Y   Y   C   E   G   E   C   A   F   P   L
                80                              90
N   S   Y   M   N   A   T   N   H   A   I   V   Q   T   L
                            100
V   H   F   I   N   P   E   T   V   P   K   P   C   C   A
            110                             120
P   T   Q   L   N   A   I   S   V   L   Y   F   D   D   S
                            130
S   N   V   I   L   K   K   Y   R   N   M   V   V   R   A

C   G   C   H.
```


        Both the pro form and prepro form, when
properly dimerized, folded, adsorbed on a matrix, and
implanted, display osteogenic activity, presumably
due to proteolytic degradation resulting in cleavage
and generation of mature form protein or active
truncated analogs.

Active OP1 can also be purified in a
truncated form, missing part of the protein's N
terminus.  One active truncated form of OP1 is (Seq.
ID No. 2):

OP1-16S

```
                              8     10
                              S  Q  N  R  S  K  T  P
                     20                            30
         K  N  Q  E  A  L  R  M  A  N  V  A  E  N  S
                                    40
         S  S  D  Q  R  Q  A  C  K  K  H  E  L  Y  V
                     50                            60
         S  F  R  D  L  G  W  Q  D  W  I  I  A  P  E
                                    70
         G  Y  A  A  Y  Y  C  E  G  E  C  A  F  P  L
                     80                            90
         N  S  Y  M  N  A  T  N  H  A  I  V  Q  T  L
                                    100
         V  H  F  I  N  P  E  T  V  P  K  P  C  C  A
                     110                          120
         P  T  Q  L  N  A  I  S  V  L  Y  F  D  D  S
                                    130
         S  N  V  I  L  K  K  Y  R  N  M  V  V  R  A

         C  G  C  H.
```

Four other active truncated forms of
OP1 are:

OP1-16L (Seq. ID. No. 3)

```
                              21                 30
                              L  R  M  A  N  V  A  E  N  S
                                    40
         S  S  D  Q  R  Q  A  C  K  K  H  E  L  Y  V
                     50                            60
         S  F  R  D  L  G  W  Q  D  W  I  I  A  P  E
                                    70
         G  Y  A  A  Y  Y  C  E  G  E  C  A  F  P  L
                     80                            90
         N  S  Y  M  N  A  T  N  H  A  I  V  Q  T  L
                                    100
         V  H  F  I  N  P  E  T  V  P  K  P  C  C  A
                     110                          120
         P  T  Q  L  N  A  I  S  V  L  Y  F  D  D  S
                                    130
         S  N  V  I  L  K  K  Y  R  N  M  V  V  R  A

         C  G  C  H;
```

<u>OP1-16M</u> (Seq. ID. No. 4)

```
                              23                          30
                              M   A   N   V   A   E   N   S
                                          40
      S   S   D   Q   R   Q   A   C   K   K   H   E   L   Y   V
                          50                              60
      S   F   R   D   L   G   W   Q   D   W   I   I   A   P   E
                                          70
      G   Y   A   A   Y   Y   C   E   G   E   C   A   F   P   L
                          80                              90
      N   S   Y   M   N   A   T   N   H   A   I   V   Q   T   L
                                          100
      V   H   F   I   N   P   E   T   V   P   K   P   C   C   A
                      110                              120
      P   T   Q   L   N   A   I   S   V   L   Y   F   D   D   S
                                      130
      S   N   V   I   L   K   K   Y   R   N   M   V   V   R   A

      C   G   C   H ;
```

<u>OP1-16A</u> (Seq. ID. No. 5)

```
                              24                          30
                              A   N   V   A   E   N   S
                                          40
      S   S   D   Q   R   Q   A   C   K   K   H   E   L   Y   V
                          50                              60
      S   F   R   D   L   G   W   Q   D   W   I   I   A   P   E
                                          70
      G   Y   A   A   Y   Y   C   E   G   E   C   A   F   P   L
                          80                              90
      N   S   Y   M   N   A   T   N   H   A   I   V   Q   T   L
                                          100
      V   H   F   I   N   P   E   T   V   P   K   P   C   C   A
                      110                              120
      P   T   Q   L   N   A   I   S   V   L   Y   F   D   D   S
                                      130
      S   N   V   I   L   K   K   Y   R   N   M   V   V   R   A

      C   G   C   H ; and
```

## OP1-16V (Seq. ID. No. 6)

```
                                    26          30
                                    V   A   E   N   S
                                40
    S   S   D   Q   R   Q   A   C   K   K   H   E   L   Y   V
            50                                              60
    S   F   R   D   L   G   W   Q   D   W   I   I   A   P   E
                                70
    G   Y   A   A   Y   Y   C   E   G   E   C   A   F   P   L
            80                                              90
    N   S   Y   M   N   A   T   N   H   A   I   V   Q   T   L
                            100
    V   H   F   I   N   P   E   T   V   P   K   P   C   C   A
            110                                            120
    P   T   Q   L   N   A   I   S   V   L   Y   F   D   D   S
                            130
    S   N   V   I   L   K   K   Y   R   N   M   V   V   R   A

    C   G   C   H .
```

Given the foregoing amino acid and DNA sequence information, various DNAs can be constructed which encode at least the minimal active domain of OP1, and various analogs thereof, as well as fusion proteins, other truncated forms of the mature proteins, and similar constructs. These DNAs can be produced by those skilled in the art using well known DNA manipulation techniques involving genomic and cDNA isolation, construction of synthetic DNA from synthesized oligonucleotides, and cassette mutagenesis techniques. 15-100mer oligonucleotides may be synthesized on a Biosearch DNA Model 8600 Synthesizer, and purified by polyacrylamide gel electrophoresis (PAGE) in Tris-Borate-EDTA buffer. The DNA may then be electroeluted from the gel. Overlapping oligomers may be phosphorylated by T4 polynucleotide kinase and ligated into larger blocks which may also be purified by PAGE.

The cDNA or synthetic DNA then may be
integrated into an expression vector and transfected
into an appropriate host cell for protein
expression.  The host may be a procaryotic or
eucaryotic cell since the former's inability to
glycosylate protein will not destroy the protein's
osteogenic activity.  Useful host cells include E.
coli, Saccharomyces, the insect/baculovirus cell
system, myeloma cells, and various mammalian cells.
The protein of this invention preferably is expressed
in mammalian cells, as disclosed herein.  The vector
additionally may encode various sequences to promote
correct expression of the recombinant protein,
including transcription promoter and termination
sequences, enhancer sequences, preferred ribosome
binding site sequences, preferred mRNA leader
sequences, preferred signal sequences for protein
secretion, and the like.  The DNA sequence encoding
the gene of interest also may be manipulated to
remove potentially inhibiting sequences or to
minimize unwanted secondary structure formation.  The
recombinant osteogenic protein also may be expressed
as a fusion protein.  After being translated, the
protein may be purified from the cells themselves or
recovered from the culture medium.  All biologically
active protein forms comprise dimeric species joined
by disulfide bonds or otherwise associated, produced
by oxidizing and refolding one or more of the various
recombinant proteins within an appropriate eucaryotic
cell or in vitro after expression of individual
subunits.

As stated earlier, it is generally held that
recombinant production of mammalian proteins for

therapeutic uses are preferably expressed in
mammalian cell culture systems in order to produce a
protein whose structure is most like that of the
natural material.  Recombinant protein production in
mammalian cells requires the establishment of
appropriate cells and cell lines that are easy to
transfect, are capable of stably maintaining foreign
DNA with an unrearranged sequence, and which have the
necessary cellular components for efficient
transcription, translation, post-translation
modification, and secretion of the protein.  In
addition, a suitable vector carrying the gene of
interest also is necessary.  DNA vector design for
transfection into mammalian cells should include
appropriate sequences to promote expression of the
gene of interest as described supra, including
appropriate transcription initiation, termination,
and enhancer sequences, as well as sequences that
enhance translation efficiency, such as the Kosak
consensus sequence.  Preferred DNA vectors also
include a marker gene and means of amplifying the
copy number of the gene of interest.

Substantial progress in the development of
mammalian cell expression systems has been made in
the last decade and many aspects of the system are
well characterized.  A detailed review of the state
of the art of the production of foreign proteins in
mammalian cells, including useful cells, protein
expression-promoting sequences, marker genes, and
gene amplification methods, is disclosed in Bendig,
Mary M., (1988) Genetic Engineering, 7:91-127.

Briefly, among the best characterized transcription promoters useful for expressing a foreign gene in a particular mammalian cell are the SV40 early promoter, the adenovirus promoter (AdMLP), the mouse metallothionein-I promoter (mMT-I), the Rous sarcoma virus (RSV) long terminal repeat (LTR), the mouse mammary tumor virus long terminal repeat (MMTV-LTR), and the human cytomegalovirus major intermediate-early promoter (hCMV). The DNA sequences for all of these promoters are known in the art and are available commercially.

One of the better characterized methods of gene amplification in mammalian cell systems is the use of the inducible DHFR gene in a dhfr- cell line. Generally, the DHFR gene is provided on the vector carrying the gene of interest, and induction by addition of the cytotoxic drug methotrexate amplifies the DHFR gene copy number, as well as that of the associated gene of interest. DHFR as an inducible, amplifying marker gene in transfected chinese hamster ovary cell lines (CHO cells) is particularly well characterized in the art. Other genes useful as inducible gene amplifiers include the adenosine deaminase (ADA) and glutamine synthetase (GS) genes.

The choice of cells/cell lines is also important and depends on the needs of the experimenter. Monkey kidney cells (COS) provide high levels of transient gene expression, providing a useful means for rapidly testing vector construction and the expression of cloned genes. COS cells are transfected with a simian virus 40 (SV40) vector carrying the gene of interest. The transfected COS

cells eventually die, thus preventing the long term
production of the desired protein product. However,
transient expression does not require the time
consuming process (often several weeks) required for
the development of a stable cell line.

Among established cell lines, CHO cells may
be the best characterized to date. They also are
capable of expressing proteins from a broad range of
cell types. The general applicability of CHO cells
and its successful production for a wide variety of
human proteins in unrelated cell types emphasizes the
underlying similarity of all mammalian cells. Thus,
while the glycosylation pattern on a recombinant
protein produced in a mammalian cell expression
system may not be identical to the natural protein,
the differences in oligosaccharide side chains are
often not essential for biological activity of the
expressed protein.

Methods for expressing and purifying
recombinant OP1 from a variety of mammalian cells,
the nature of the xenogenic matrix, and other
material aspects concerning the nature, utility, and
how to make and how to use the subject matter claimed
will be further understood from the following, which
constitutes the best method currently known for
practicing the invention.

I.  RECOMBINANT PROTEIN EXPRESSION
    IN MAMMALIAN CELLS

Several different mammalian cell expression
systems have been used to express recombinant OP1

proteins of this invention. In particular, COS cells
are used for the rapid assessment of vector
construction and gene expression, using an SV40
vector to transfect the DNA sequence into COS cells.
Stable cell lines are developed using CHO cells
(chinese hamster ovary cells) and a
temperature-sensitive strain of BSC cells (simian
kidney cells, BSC40-tsA58, (1988) Biotechnology 6:
1197-1196) for the long term production of OP1. Two
different promoters are used to transcribe OP1: the
CMV promoter, boosted by the enhancer sequence from
the Rous sarcoma virus LTR, and the mMT promoter
(mouse metallothionein promoter). Several selection
marker genes also are used, namely, neo (neomycin)
and DHFR. The DHFR gene also may be used as part of
a gene amplification scheme for CHO cells. Another
gene amplification scheme relies on the temperature
sensitivity (ts) of BSC40-tsA58 cells transfected
with an SV40 vector. Temperature reduction to 33°C
stabilizes the ts SV40 T antigen which leads to the
excision and amplification of the integrated
transfected vector DNA, thereby also amplifying the
associated gene of interest.

Stable cell lines were established for CHO
cells as well as BSC40-tsA58 cells (hereinafter
referred to as "BSC cells"). The various cells, cell
lines and DNA sequences chosen for mammalian cell
expression of the OP1 proteins of this invention are
well characterized in the art and are readily
available. Other promoters, selectable markers, gene
amplification methods and cells also may be used to
express the OP1 proteins of this invention, as well
as other osteogenic proteins. Particular details of

the transfection, expression, and purification of
recombinant proteins are well documented in the art
and are understood by those having ordinary skill in
the art.  Further details on the various technical
aspects of each of the steps used in recombinant
production of foreign genes in mammalian cell
expression systems can be found in a number of texts
and laboratory manuals in the art, such as, for
example, <u>Current Protocols in Molecular Biology</u>, F.M.
Ausubel et al., ed., John Wiley & Sons, New York 1987.


1.   Exemplary Expression Vectors


     Figure 3 discloses restriction maps of
various exemplary expression vectors designed for OP1
expression in mammalian cells.  Each of these vector
contructs employs a full-length cDNA sequence
originally isolated from a human cDNA library (human
placenta) and subsequently cloned into a conventional
pUC vector (pUC-18) using pUC polylinker sequences at
the insertion sites.  The OP1 cDNA fragment cloned
into each of these constructs is either the intact
SmaI-BamHI OP1 cDNA fragment depicted in Figure 2
(Seq. ID No.7), or modifications of this fragment
where the flanking non-coding 5' and/or 3' sequences
have been trimmed back, using standard molecular
biology methodology.  Each vector also employs an
SV40 origin of replication (ori), useful for
mediating plasmid replication in primate cells (e.g.,
COS and BSC cells).  In addition, the early SV40
promoter is used to drive transcription of marker
genes on the vector (e.g., neo and DHFR).


     The pH717 expression vector (Fig. 3A)
contains the neomycin (neo) gene as an inducible

selection marker.  This marker gene is well
characterized in the art and is available
commercially.  Alternatively, other selectable
markers may be used.  The particular vector used to
provide the neo gene DNA fragment for pH717 may be
obtained from Clontech, Inc., Palo Alto, CA
(pMAM-neo-blue).  In pH717, OP1 DNA transcription is
driven by the CMV promoter, boosted by the RSV-LTR
and MMTV-LTR (mouse mammary tumor virus) enhancer
sequences.  These sequences are known in the art, and
are available commercially.  For example, vectors
containing this promoter/enhancer sequence may be
obtained from Invitrogen Inc., San Diego, CA, (e.g.,
pCDM8).

        Expression vector pH731 (Fig.3B), utilizes
the SV40 late promoter to drive OP1 transcription.
As indicated above, the sequence and characteristics
of this promoter also are well known in the art.
Alternatively, pH731 may be generated by inserting
the SmaI-BamHI fragment of OP1 into pEUK-C1
(Clontech, Inc., Palo Alto, CA).

        The pH754 expression vector (Fig. 3C)
contains the DHFR sequence as both a selection marker
and as an inducible gene amplifier.  OP1 is under CMV
control.  The DNA sequence for DHFR and is well
characterized in the art, and is available
commercially.  Alternatively, pH754 may be generated
from pMAM-neo (Clontech, Inc., Palo Alto, CA) by
replacing the neo gene (BamHI digest) with a BamHI
fragment containing the DHFR gene (e.g., obtained
from pSV5-dhfr (ATCC #37148)).  OP1 DNA then can be
inserted into the polylinker site downstream of the
MMTVLTR sequence (mouse mammary tumor virus LTR),

yielding pH752 (Fig. 3D).  The CMV promoter sequence
then may be inserted into pH752 (opened at Clal-Nhel)
as a Clal-Xbal fragment (e.g., from pMAM-neo blue,
Clontech, Inc.).

          The pW24 vector (Fig. 3E), is essentially
identical in sequence to p754, except that neo is
used as the marker gene (see pH717), in place of DHFR.

          Similarly, pH783 (Fig. 3F) contains the
amplifiable marker DHFR, but here OP1 is under mMT
(mouse metallothionein promoter) control.  The mMT
promoter is well characterized in the art and is
available commercially.  Alternatively, a Clal-Nhel
fragment containing the mMT promoter sequence
(available from Allegro Nichols Institute
Diagnostics, San Juan Capistrano, CA) can be inserted
into pH752 to generate pH783.

          All vectors tested are stable in the various
cells used to express OP1, and provide a range of OP1
expression levels.

2.   Exemplary Mammalian Cells

          Recombinant OP1 has been expressed in three
different cell expression systems:  COS cells for
rapidly screening the functionality of the various
expression vector constructs, CHO cells for the
establishment of stable cell lines, and BSC40-tsA58
cells as an alternative means of producing OP1
protein.

A.    COS CELLS

COS cells (simian kidney cells) are used for rapid screening of vector contructs and for immediate, small scale production of OP1 protein. COS cells are well known in the art and are available commercially.  The particular cell line described herein may be obtained through the American Type Culture Collection (ATCC #COS-1, CRL-1650).

OP1 expression levels from different vectors, analyzed by northern and western blot assays, are compared in Table I below:

TABLE 1

ANALYSIS OF OP1 EXPRESSION IN COS CELLS

| Vector | mRNA | OP1 Production |
|--------|------|----------------|
| pH717 | +++ | ++ |
| pH731 | + | + |
| pH752 | +++ | ++++ |
| pH754 | +++ | ++++ |

pH754-transfected COS cells appear to produce the highest yield of OP1 to date.  However, because transfected COS cells do not divide and die several days post-transfection, large amounts of plasmid DNA are required for each scaled up transformation.

Large scale preparations of OP1 from transfected COS cells may be produced using conventional roller bottle technology.  Briefly, 14 X

$10^6$ cells are used to seed each bottle. After 24 hrs
of growth, the cells are transfected with 10 μg of
vector DNA (e.g., pH717) per $10^6$ cells, using the
DEAE-dextran method. Cells are then conditioned in
serum-free media for 120 hr before harvesting the
media for protein analysis. Following this protocol,
OP1 yield is approximately 2-6 ng/ml.

B.    CHO Cells

CHO cells (chinese hamster ovary cells) may
be used for long term OP1 production. CHO cell lines
are well characterized for the small and large scale
production of foreign genes and are available
commercially. The particular cell line described
herein is CHO-DXB11, (Laurence Chasin, Columbia
University, NY). Table II, below, shows exemplary
OP1 yields obtained with a variety of expression
vectors.

## TABLE II

| CHO Cells | Plasmid | Selection Marker | OP1 Production ng/ml |
|---|---|---|---|
| | pH717 | NEO | 2-5 |
| * | pH752/pH754 | DHFR | 100-150 |

*Cells are adapted to grow in
0.1 uM methotrexate

CHO cells may be transfected by conventional
calcium phosphate technique. CHO cells preferably
are transfected with pH754 or pH752 and are

conditioned in media containing serum proteins, as
this appears to enhance OP1 yields. Useful media
include media containing 0.1-0.5% dialyzed fetal calf
serum (FCS).


C.   BSC CELLS


The BSC40-tsA58 cell line ("BSC cells") is a
temperature-sensitive strain of simian kidney cells
(1988, <u>Biotechnology 6</u>: 1192-1196) which overcomes
some of the problems associated with COS cells.
These BSC cells have the advantage of being able to
amplify gene sequences rapidly on a large scale with
temperature downshift, without requiring the addition
of exogenous, potentially toxic drugs.  In addition,
the cells may be recycled.  That is, after induction
and stimulation of OP1 expression, the cells may be
transferred to new growth medium, grown to confluence
at 39.5°C and induced a second time by downshifting
the temperature to 33°C.  BSC cells may be used to
establish stable cell lines rapidly for protein
production.


Transfected BSC cells may be induced by
shifting the temperature down to 33°C, in media
containing 10% FCS, and harvesting the conditioned
media after 96 hrs of incubation.  Comparable amounts
of OP1 RNA and protein are obtained, as compared with
CHO cells (e.g., 100-150 ng OP1/ml conditioned media
from BSC clones transfected with pH717).


3.        Evaluation of OP1 transfected cells

Expression levels of transfected OP1
sequences can be measured in the different systems by
analyzing mRNA levels (Northern blots), using total
cellular RNA and conventional hybridization
methodology. Generally, about $1 \times 10^6$ cells are
needed for mRNA analysis. Data between individual
cell lines can be compared if the total number of
cells and the total amount of mRNA is normalized,
using rRNA as an internal standard. Ribosomal RNA is
visualized in the agarose gel by ethydium bromide
stain prior to transfer of the RNA to nitrocellulose
sheets for hybridization. Ribosomal RNA also
provides an indicator of the integrity of the RNA
preparation.

OP1 protein levels also may be measured by
Western blots (immunoblots) using rabbit antisera
against human OP1. Figure 4 is an immunoblot showing
OP1 production in: COS cells - (A) pH717, (B)
pH731; CHO cells - (C) pH754, (D) pH752; and BSC
cells - (E) pH717 and (F) pW24.

Southern blots may be used to assess the
state of integrated OP1 sequences and the extent of
their copy number amplification. The copy number of
excised plasmids in temperature-shifted BSC cells
also can be determined using Southern blot analysis.


II.  PROTEIN PURIFICATION


The purification scheme developed to purify
the recombinant osteogenic proteins of this invention
is rapid and highly effective. The protocol involves

three chromatographic steps (S-Sepharose, phenyl-Sepharose and C-18 HPLC), and produces OP1 of about 90% purity.

For a typical 2 L preparation of transfected BSC cells conditioned in 0.5% FCS, the total protein is 700 mg. The amount of OP1 in the media, estimated by western blot, is about 80 μg. OP1 media is diluted to 6M urea, 0.05M NaCl, 13mM HEPES, pH 7.0 and loaded onto an S-Sepharose column, which has attached sulfite groups and acts as a strong cation exchanger. OP1 binds to the column in low salt, and serum proteins are removed. The column is subsequently developed with two step salt elutions. The first elution (0.1M NaCl) removes contaminants and approximately 10% of the bound OP1. The remaining 90% of OP1 then is eluted in 6M urea, 0.3M NaCl, 20mM HEPES, pH 7.0.

Ammonium sulfate is added to the 0.3M NaCl fraction to obtain final solution conditions of 6M urea, 1M $(NH_4)_2SO_4$, 0.3M NaCl, 20mM HEPES, pH 7.0. The sample then is loaded onto a phenyl-Sepharose column (hydrophobic interaction chromatography). OP1 binds phenyl-Sepharose in the presence of high concentrations of a weak chaotropic salt (e.g., 1M $(NH_4)_2SO_4$). Once OP1 is bound, the column is developed with two step elutions using decreasing concentrations of ammonium sulfate. The first elution (containing 0.6M $(NH_4)_2SO_4$) primarily removes contaminants. The bound OP1 then is eluted with a 6M urea, 0.3M NaCl, 20MM HEPES, pH 7.0 buffer containing no ammonium sulfate.

The OP1 eluted from the phenyl-Sepharose
column is dialyzed against water, followed by 30%
acetonitrile (0.1% TFA), and then applied to a C-18
reverse phase HPLC column. Figures 5A, B, and C are
(1) chromatograms and (2) coomassie-stained SDS-PAGE
gels of fractions after reduction with dithiothreitol
(DTT) eluting from the (A) S-Sepharose, (B)
phenyl-Sepharose, and (C) C-18 columns. Gel
separation of oxidized and reduced OP1 samples show
that the reduced subunit has an apparent molecular
weight of about 18 kD, and the dimer has an apparent
molecular weight of about 36 kD, as illustrated in
Figure 6. The subunit size appears to be identical
to that purified from COS cells, as well as that of
the naturally-sourced bOP. The current protocol
yields about 30 ug of OP1 for 2 L of conditioned
media, a recovery of about 25%, as estimated by gel
scanning.

An alternative chromatography protocol is to
perform the S-Sepharose chromatography in the absence
of 6 M urea. The bound proteins then are eluted with
salt step elutions (e.g., 100-400 mM NaCl). Most of
the OP1 is eluted with about 300 mM NaCl. Additional
OP1 then can be eluted with 300 mM MaCl in the
presence of 6M urea. The 6M urea elution also may be
used in place of the non-urea elution to achieve
maximum recovery in one step.

OP1 also will bind hydroxyapatite
efficiently, but only in the absence of 6 M urea and
at low phosphate concentrations (less than 5 mM
phosphate). Bound OP1 can be removed from the column
with a step elution of 1 mM to 0.5M phosphate (in 0.5

M NaCl, 50 mM Tris, pH 7.0). OP1 elutes at about 250
mM phosphate. Additionally, urea (6M) may be added
during the elution step.

Other related chromatography methods also
may be useful in purifying OP1 from eukaryotic cell
culture systems. For example, heparin-Sepharose may
be used in combination with the S-Sepharose column.
Alternatively, $Cu^{2+}$-immobilized metal-ion affinity
chromatography (IMAC) will bind OP1 in a phosphate
buffer (pH7.0) containing 6M urea.

## III. MATRIX PREPARATION

Practice of the invention requires the
availability of bone, preferably mammalian bone,
e.g., bovine. The bone is cleaned, demarrowed,
delipidated, demineralized, reduced to particles of
an appropriate size, extracted to remove soluble
proteins, sterilized, and otherwise treated as
disclosed herein to produce an implantable material
useful in a variety of clinical settings.

Matrices of various shapes fabricated from
the material of the invention may be implanted
surgically for various purposes. Chief among these
is to serve as a matrix for bone formation in various
orthopedic, periodontal, and reconstructive
procedures, as a sustained release carrier, or as a
collagenous coating for implants. The matrix may be
shaped as desired in anticipation of surgery or
shaped by the physician or technician during
surgery. Thus, the material may be used for topical,
subcutaneous, intraperitoneal, or intramuscular

implants; it may be shaped to span a nonunion fracture or to fill a bone defect. In bone formation or conduction procedures, the material is slowly absorbed by the body and is replaced by bone in the shape of or very nearly the shape of the implant.

Various growth factors, hormones, enzymes, therapeutic compositions, antibiotics, and other body treating agents also may be sorbed onto the carrier material and will be released over time when implanted as the matrix material is slowly absorbed. Thus, various known growth factors such as EGF, PDGF, IGF, FGF, TGF alpha, and TGF beta may be released _in vivo_. The material can be used to release chemotherapeutic agents, insulin, enzymes, or enzyme inhibitors.

Details of how to make and how to use the materials of the invention are disclosed below.

1.         <u>Preparation of Demineralized Bone</u>

Demineralized bovine bone matrix is prepared by previously published procedures (Sampath and Reddi (1983) Proc. Natl. Acad. Sci. USA <u>80</u>:6591-6595). Bovine diaphyseal bones (age 1-10 days) are obtained from a local slaughterhouse and used fresh. The bones are stripped of muscle and fat, cleaned of periosteum, demarrowed by pressure with cold water, dipped in cold absolute ethanol, and stored at -20°C. They are then dried and fragmented by crushing and pulverized in a large mill. Care is

taken to prevent heating by using liquid nitrogen.
The pulverized bone is milled to a particle size in
the range of 70-850 μm, preferably 150 μm-420 μm, and
is defatted by two washes of approximately two hours
duration with three volumes of chloroform and
methanol (3:1). The particulate bone is then washed
with one volume of absolute ethanol and dried over
one volume of anhydrous ether yielding defatted bone
powder. The defatted bone powder is then
demineralized by four successive treatments with 10
volumes of 0.5 N HCl at 4°C for 40 min. Finally,
neutralizing washes are done on the demineralized
bone powder with a large volume of water.

2.     Guanidine Extraction

        Demineralized bone matrix thus prepared is
extracted with 5 volumes of 4 M guanidine-HCl, 50mM
Tris-HCl, pH 7.0 for 16 hr. at 4°C. The suspension
is filtered. The insoluble material is collected and
used to fabricate the matrix. The material is mostly
collagenous in nature. It is devoid of osteogenic or
condrogenic activity.

3.     Matrix Treatments

        The major component of all bone matrices is
Type-I collagen. In addition to collagen,
demineralized bone extracted as disclosed above
includes non-collagenous proteins which may account
for 5% of its mass. In a xenogenic matrix, these
noncollagenous components may present themselves as
potent antigens, and may constitute immunogenic
and/or inhibitory components. These components also

may inhibit osteogenesis in allogenic implants by
interfering with the developmental cascade of bone
differentiation.  It has been discovered that
treatment of the matrix particles with a collagen
fibril-modifying agent extracts potentially unwanted
components from the matrix, and alters the surface
structure of the matrix material.

The currently most preferred fibril
modifying agent is a heated aqueous medium, most
preferably water.  Various amounts of delipidated,
demineralized guanidine-extracted bone collagen is
heated in water (1 g/30 ml) under constant stirring
in a glass flask, water jacked, and maintained at a
given temperature for 1 hour.  In some instances the
water may be replaced with 0.1M acetic acid to help
"swell" the collagen before heating.  The temperature
employed is held constant at room temperature, and
about 37°C, 45°C, 55°, 65°, 75°.  After the heat
treatment, the matrix is filtered and lyophilized and
used for implant.

The effects of hot water treatment on
morphology of the matrix material is apparent from a
comparison of the photomicrographs in Figure 6 with
those of Figure 1.  Figure 6 illustrates the
morphology of the successfully altered collagen
surface treated at (a) 37°C, (b) 45°C, (c) 55°C and
(d) 65°C.  The photomicrographs of Figure 1 describe
the morphology of untreated rat and bovine bone
matrix (A and B, respectively).  As is evident from
the micrographs, the hot water treatment increases
the degree of micropitting on the particle surface
at least about 10-fold, as well as also substantially

increasing particle's porosity (compare Figure 1B and
5C, 5D). This alteration of the matrix particle's
morphology substantially increases the particle
surface area. Careful measurement of the pore and
micropit sizes reveals that hot aqueous medium
treatment of the matrix particles yields particle
pore and micropit diameters within the range of 1μm
to 100μm.

Characterization of the extract produced by
the hot water treatment reveals that the treatment
also may be removing component(s) whose association
with the matrix may interfere with new bone formation
in vivo. Figure 8 is a 214 nm absorbance tracing
of the extract isolated from hot water treated bovine
matrix, and indicates the effect of each peak (or
fraction) on in vivo bone formation.

The extract from a large scale preparative
run (100 g bovine matrix, hot water-treated) was
collected, acidified with 0.1% TFA, and run on a C-18
HPLC column, using a Millipore Delta Prep Cartridge.
Fractions were collected at 50 mL intervals at a flow
rate of 25 ml/min. and pooled appropriately to
isolate the individual peaks in the tracing. Each of
these fractions then was implanted with recombinant
OP1 and an appropriate rat matrix carrier (see
infra), and its effect on bone formation activity
measured. Fraction 12 alone appears to inhibit bone
formation in allogenic implants. The inhibitory
activity appears to be dose dependent. It is
possible that the removal of the inhibitory
component(s) present in this peak may be necessary to
support osteogenic activity in xenogenic implants.

Figure 9 describes the influence of complete
solvent extract from hot water-treated matrix on
osteogenic activity as measured in 12-day implants,
and determined by alkaline phosphatase activity and
calcium content. Rat carrier matrix and OP1
implanted without any extract is used as a positive
control. The solvent extract obtained from 100 grams
of hot water-treated bovine matrix was evaporated and
taken up in 6 M of 50% acetonitrile/0.1% TFA.
100-300 µl aliquots then were combined with known
amounts of recombinant OP1, and 25 mg of rat matrix
carrier, and assayed (see infra). The results
clearly show the extract inhibits new bone formation
in a dose dependent manner.

After contact with the fibril-modifying
agent, the treated matrix is washed to remove any
extracted components, following a form of the
procedure set forth below:

1.    Suspend in TBS (Tris-buffered saline)
1g/200 ml and stir at 4°C for 2 hrs; or in 6 M urea,
50 mM Tris-HCl, 500 mM NACl, pH 7.0 (UTBS) or water
and stir at room temperature (RT) for 30 minutes
(sufficient time to neutralize the pH);

2.    Centrifuge and repeat wash step; and

3.    Centrifuge; discard supernatant; water
wash residue; and then lyophilize.

Other useful fibril-modifying agents include
acids such as trifluoroacetic acid and hydrogen

fluoride, and organic solvents such as
dichloromethane, acetonitrile, isopropanol, and
chloroform, as well as combinations of these agents.
Matrix treatments using these other fibril-modifying
agents, as well as a detailed physical analysis of
the effect these fibril-modifying agents have on
demineralized, guanidine-extracted bone collagen
particles is disclosed in copending U.S. Patent
Application No. 422,613, filed 10/17/89, the
disclosure of which is hereby incorporated by
reference.

The collagen matrix materials preferably
take the form of a fine powder, insoluble in water,
comprising nonadherent particles.  It may be used
simply by packing into the volume where new bone
growth or sustained release is desired, held in place
by surrounding tissue.  Alternatively, the powder may
be encapsulated in, e.g., a gelatin or polylactic
acid coating, which is adsorbed readily by the body.
The powder may be shaped to a volume of given
dimensions and held in that shape by interadhering
the particles using, for example, soluble, species
biocompatible collagen.  The material may also be
produced in sheet, rod, bead, or other macroscopic
shapes.

IV.   FABRICATION OF OSTEOGENIC DEVICE

The recombinant protein as set forth above,
and other constructs, can be combined and dispersed
in a suitable matrix preparation using any of the
methods described below:

1.        Ethanol Precipitation


     Matrix is added to osteogenic protein
dissolved in guanidine-HCl.  Samples are vortexed and
incubated at a low temperature.  Samples are then
further vortexed.  Cold absolute ethanol is added to
the mixture which is then stirred and incubated.
After centrifugation (microfuge, high speed) the
supernatant is discarded.  The matrix is washed with
cold concentrated ethanol in water and then
lyophilized.


2.        Acetonitrile Trifluoroacetic
          Acid Lyophilization


     In this procedure, osteogenic protein in an
acetonitrile trifluroacetic acid (ACN/TFA) solution
was added to the carrier material.  Samples were
vigorously vortexed many times and then lyophilized.
Osteogenic protein was added in varying
concentrations, and at several levels of purity.
This method is currently preferred.


3.        Urea Lyophilization


     For those osteogenic proteins that are
prepared in urea buffer, the protein is mixed with
the matrix material, vortexed many times, and then
lyophilized.  The lyophilized material may be used
"as is" for implants.

4.       Buffered Saline Lyophilization

OP preparations in physiological saline may
also be vortexed with the matrix and lyophilized to
produce osteogenically active material.

These procedures also can be used to adsorb
other active therapeutic drugs, hormones, and various
bioactive species for sustained release purposes.

## V.   BIOASSAY

The functioning of the various matrices can
be evaluated with an _in vivo_ rat bioassay.  Studies
in rats show the osteogenic effect in an appropriate
matrix to be dependent on the dose of osteogenic
protein dispersed in the matrix.  No activity is
observed if the matrix is implanted alone.
Demineralized, guanidine extracted xenogenic bone
matrix materials of the type described in the
literature are ineffective as a carrier, fail to
induce bone, and produce an inflammatory and
immunological response when implanted unless treated
as disclosed above.  Many of the allogenic matrix
materials also are ineffective as carriers.  The
following sets forth various procedures for preparing
osteogenic devices from control and matrix materials
prepared as set forth above, and for evaluating their
osteogenic utility.

## Implantation

The bioassay for bone induction as described
by Sampath and Reddi (Proc. Natl. Acad. Sci. USA
(1983) 80: 6591-6595), herein incorporated by
reference, may be used to monitor endochondral bone

differentiation activity. This assay consists of implanting the bovine test samples xenogenically in subcutaneous sites in recipient rats under ether anesthesia. Male Long-Evans rats, aged 28-32 days, were used. A vertical incision (1 cm) is made under sterile conditions in the skin over the thoraic region, and a pocket is prepared by blunt dissection. Approximately 25 mg of the test sample is implanted deep into the pocket and the incision is closed with a metallic skin clip. The day of implantation is designated as day of the experiment. Implants were removed on day 12. The heterotropic site allows for the study of bone induction without the possible ambiguities resulting from the use of orthotropic sites.

## Cellular Events

Successful implants exhibit a controlled progression through the stages of matrix induced endochondral bone development including: (1) transient infiltration by polymorphonuclear leukocytes on day one; (2) mesenchymal cell migration and proliferation on days two and three; (3) chondrocyte appearance on days five and six; (4) cartilage matrix formation on day seven; (5) cartilage calcification on day eight; (6) vascular invasion, appearance of osteoblasts, and formation of new bone on days nine and ten; (7) appearance of osteoblastic and bone remodeling and dissolution of the implanted matrix on days twelve to eighteen; and (8) hematopoietic bone marrow differentiation in the ossicle on day twenty-one. The results show that the shape of the new bone conforms to the shape of the implanted matrix.

## Histological Evaluation

Histological sectioning and staining is
preferred to determine the extent of osteogenesis in
the implants.  Implants are fixed in Bouins Solution,
embedded in paraffin, and cut into 6-8 μm sections.
Staining with toluidine blue or hemotoxylin/eosin
demonstrates clearly the ultimate development of
endochondral bone.  Twelve day implants are usually
sufficient to determine whether the implants contain
newly induced bone.

## Biological Markers

Alkaline phosphatase activity may be used as
a marker for osteogenesis.  The enzyme activity may
be determined spectrophotometrically
after homogenization of the implant.  The activity
peaks at 9-10 days _in vivo_ and thereafter slowly
declines.  Implants showing no bone development by
histology have little or no alkaline phosphatase
activity under these assay conditions.  The assay is
useful for quantitation and obtaining an estimate of
bone formation quickly after the implants are removed
from the rat.  Alternatively, the amount of bone
formation can be determined by measuring the calcium
content of the implant.

## Results

OP1 from different cell sources and purified
to different extents (1-5% pure to 30-90% pure) were
tested for osteogenic activity _in vivo_ as set forth

above using matrices of approximately 25 mg. Table
III below shows the histology score for OP1 expressed
in all three cell types.


TABLE III

| Mammalian Cells | OP1 Subunit | Protein Concentration† (ng) | Histology Score (%) |
|---|---|---|---|
| BSC40-tsA58 | 18kDa* | 32.5 | 50 |
|  |  | 65.0 | 40 |
|  |  | 130.0 | 80 |
|  |  | 260.0 | 100 |
|  | 16 kDa+ | 12.5 | 20 |
|  |  | 25.0 | 50 |
|  |  | 50.0 | 80 |
|  |  | 100.0 | 100 |
|  |  | 200.0 | 100 |
| CHO | 16-20 kDa$^S$ | 50.0 | 90 |
|  |  | 100.0 | 90 |
|  |  | 200.0 | 100 |
| COS | 18 kDa$^S$ | 25.0 | 10 |
|  |  | 50.0 | 30 |
|  |  | 100.0 | 90 |
|  |  | 200.0 | 90 |

10-30%:  moderate bone formation

30-80%:  extensive bone formation

above 80%:  showed sign of hemopoietic bone
            marrow recruitment.


*  70-90% pure

+  30-40% pure

$^S$  less than 5% pure

†  estimated by immunoblots or gel scanning


The histology scores detailed in Table III
show that OP1 is active regardless of cell source,

and that the activity mimics that of native bovine
OP. The bone-inducing activity is highly
reproducible and dose dependent. Further evidence of
the bone-forming activity of recombinant OP1 is
provided in the photomicrographs of Figures 10 and 11.

Figure 10A-F are photomicrographs recording
the histology of allogenic implants using recombinant
OP1 expressed from COS, BSC, and COS cells. The
micrographs (magnified 220X), provide graphic
evidence of the full developmental cascade induced by
the osteogenic proteins of this invention, confirming
that recombinantly produced OP1 alone is sufficient
to induce endochondral bone formation, when implanted
in association with a matrix. As evidenced in Figure
10A, allogenic implants that do not contain OP1 show
no new bone formation at 12 days' post implant. Only
the implanted bone matrix (m) and surrounding
mesenchyme are seen. Conversely, implants containing
OP1 already show evidence of extensive chodrogenesis
by 7 days post implant (Fig. 10B, 500 ng BSC-produced
protein, 30% pure). Here, newly formed cartilage
cells, chrondroblasts (Cb) and chondrocytes (Cy) are
in close contact with the matrix (m). By 9 days post
implant endochondral bone differentiation, cartilage
calcification, hypertrophy of chondrocytes, vascular
invasion, and the onset of new bone formation are all
evident (Fig. 10C, 220 ng COS-produced protein,
approx. 5% pure). Invading capillaries (c) and the
appearance of basophilic osteoblasts (indicated by
arrows) near the vascular endothelium are
particularly evident. By 12 days post implant
extensive bone formation and remodeling has occurred
(Fig. 10D (220X), and 10E (400X), CHO-produced

protein, approx. 60% pure). The newly formed bone
laid down by osteoblasts is being remodeled by
multinucleated osteoclasts (Oc), and the implanted
matrix is being resorbed and replaced by remodeled
bone. Bone marrow recruitment in the newly formed
ossicles is also evident. Finally, hematopoietic
bone marrow differentiation within the ossicles can
be seen by 22 days' post implant (Fig. 10F, 500 ng
BSC-produced protein, 30% pure). By this time most
of the implanted matrix (m) has been resorbed and is
occupied by newly-formed bone containing ossicles
filled with bone marrow elements including
erythrocytic and granulocytic series and
megakaryocytes. Similar histological observations
have been made for implants incorporating greater
than 90% pure OP1 preparations.

Figure 11 is a photomicrograph showing the
histology at 12 days post implant for a xenogenic
implant using hot water-treated bovine matrix and OP1
(BSC-produced). The recruitment of hematopoietic
bone marrow elements is evident in the
photomicrograph, showing that the bone forming
activity of xenogenic implants with OP1 parallels
that of allogenic implants (compare Figure 11 with
Figures 10D and 10E).

The cellular events exhibited by the OP1
matrix implants and evidenced in Figures 10 and 11
truly mimics the endochondral bone differentiation
that occurs during the foetal development. Although
endochondral bone differentiation has been the
predominant route, there is also evidence for
intra-membraneous bone formation at the outer surface
of the implant.

Figures 12 and 13 describe the dose
dependence of osteogenic activity for 12-day
implants, as determined by specific activity of
alkaline phosphatase and calcium content of allogenic
implants (Figure 12) and xenogenic implants of this
invention (Figure 13). In all cases, OP1 protein
concentration (quantitated by immuno blot staining or
by gel scanning), is represented in nanograms. In
each case, bone inducing activity is specific to OP1
in a dose dependent manner in all cells.

The invention may be embodied in other
specific forms without departing from the spirit or
essential characteristics thereof. The present
embodiments are therefore to be considered in all
respects as illustrative and not restrictive, the
scope of the invention being indicated by the
appended claims rather than by the foregoing
description, and all changes which come within the
meaning and range of equivalency of the claims are
therefore intended to be embraced therein.

SEQUENCE LISTING

(1)   GENERAL INFORMATION:

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                           Kuberasampath, Thangavel
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      (ii)    TITLE OF INVENTION:  Osteogenic Devices
      (iii)   NUMBER OF SEQUENCE:  7
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      (v)     COMPUTER READABLE FORM:
              (A) MEDIUM TYPE:  Diskette, 3.5 inch,
                               720kb storage
              (B) COMPUTER:  IBM XT
              (C) OPERATING SYSTEM:  DOS 3.30
              (D) SOFTWARE:  Word Perfect 5.0
      (vi)    CURRENT APPLICATION DATA:
              (B) FILING DATE:  20-Aug-90
      (vii)   PRIOR APPLICATION DATA:
              (A) APPLICATION NUMBER:  US 422,699
              (B) FILING DATE:  17-Oct-89
              (C) APPLICATION NUMBER:  US 483,913
              (D) FILING DATE:  22-Feb-89

(2)   INFORMATION FOR SEQ ID NO:1:
      (i)      SEQUENCE CHARACTERISTICS:
               (A) LENGTH:  139 amino acids
               (B) TYPE:  amino acid
               (D) TOPOLOGY:  linear
      (ii)     MOLECULE TYPE:  protein
      (xi)     SEQUENCE DESCRIPTION: SEQ ID NO:1:

| Ser | Thr | Gly | Ser | Lys | Gln | Arg | Ser | Gln |
| 1 | | | | 5 | | | | |
| Asn | Arg | Ser | Lys | Thr | Pro | Lys | Asn | Gln |
| 10 | | | | | 15 | | | |
| Glu | Ala | Leu | Arg | Met | Ala | Asn | Val | Ala |
| | 20 | | | | | 25 | | |
| Glu | Asn | Ser | Ser | Ser | Asp | Gln | Arg | Gln |
| | | 30 | | | | | 35 | |
| Ala | Cys | Lys | Lys | His | Glu | Leu | Tyr | Val |
| | | | 40 | | | | | 45 |
| Ser | Phe | Arg | Asp | Leu | Gly | Trp | Gln | Asp |
| | | | | 50 | | | | |
| Trp | Ile | Ile | Ala | Pro | Glu | Gly | Tyr | Ala |
| 55 | | | | | 60 | | | |
| Ala | Tyr | Tyr | Cys | Glu | Gly | Glu | Cys | Ala |
| | 65 | | | | | 70 | | |
| Phe | Pro | Leu | Asn | Ser | Tyr | Met | Asn | Ala |
| | | 75 | | | | | 80 | |
| Thr | Asn | His | Ala | Ile | Val | Gln | Thr | Leu |
| | | | 85 | | | | | 90 |
| Val | His | Phe | Ile | Asn | Pro | Glu | Thr | Val |
| | | | | 95 | | | | |
| Pro | Lys | Pro | Cys | Cys | Ala | Pro | Thr | Gln |
| 100 | | | | | 105 | | | |
| Leu | Asn | Ala | Ile | Ser | Val | Leu | Tyr | Phe |
| | 110 | | | | | 115 | | |

```
Asp  Asp  Ser  Ser  Asn  Val  Ile  Leu  Lys
          120                           125

Lys  Tyr  Arg  Asn  Met  Val  Val  Arg  Ala
               130                           135

Cys  Gly  Cys  His.
```


(2)   INFORMATION FOR SEQ ID NO:2:
      (i)    SEQUENCE CHARACTERISTICS:
             (A) LENGTH:  132 amino acids
             (B) TYPE:  amino acid
             (D) TOPOLOGY:  linear
      (ii)   MOLECULE TYPE:  protein
      (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
                                        Ser  Gln
                                        1

Asn  Arg  Ser  Lys  Thr  Pro  Lys  Asn  Gln
          5                        10

Glu  Ala  Leu  Arg  Met  Ala  Asn  Val  Ala
               15                           20

Glu  Asn  Ser  Ser  Ser  Asp  Gln  Arg  Gln
                    25

Ala  Cys  Lys  Lys  His  Glu  Leu  Tyr  Val
30                      35

Ser  Phe  Arg  Asp  Leu  Gly  Trp  Gln  Asp
     40                        45

Trp  Ile  Ile  Ala  Pro  Glu  Gly  Tyr  Ala
          50                           55

Ala  Tyr  Tyr  Cys  Glu  Gly  Glu  Cys  Ala
               60                           65

Phe  Pro  Leu  Asn  Ser  Tyr  Met  Asn  Ala
                    70
```

```
Thr   Asn   His   Ala   Ile   Val   Gln   Thr   Leu
75                            80

Val   His   Phe   Ile   Asn   Pro   Glu   Thr   Val
      85                            90

Pro   Lys   Pro   Cys   Cys   Ala   Pro   Thr   Gln
            95                            100

Leu   Asn   Ala   Ile   Ser   Val   Leu   Tyr   Phe
                  105                            110

Asp   Asp   Ser   Ser   Asn   Val   Ile   Leu   Lys
                        115

Lys   Tyr   Arg   Asn   Met   Val   Val   Arg   Ala
120                            125

Cys   Gly   Cys   His.               .
      130
```

(2)   INFORMATION FOR SEQ ID NO:3:
        (i)     SEQUENCE CHARACTERISTICS:
                (A) LENGTH:  119 amino acids
                (B) TYPE:  amino acid
                (D) TOPOLOGY:  linear
        (ii)    MOLECULE TYPE:  protein
        (xi)    SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Leu   Arg   Met   Ala   Asn   Val   Ala   Glu   Asn
1                       5

Ser   Ser   Ser   Asp   Gln   Arg   Gln   Ala   Cys
10                      15

Lys   Lys   His   Glu   Leu   Tyr   Val   Ser   Phe
      20                      25

Arg   Asp   Leu   Gly   Trp   Gln   Asp   Trp   Ile
            30                            35

Ile   Ala   Pro   Glu   Gly   Tyr   Ala   Ala   Tyr
                  40                            45
```

```
Tyr  Cys  Glu  Gly  Glu  Cys  Ala  Phe  Pro
                    50

Leu  Asn  Ser  Tyr  Met  Asn  Ala  Thr  Asn
55                        60

His  Ala  Ile  Val  Gln  Thr  Leu  Val  His
     65                        70

Phe  Ile  Asn  Pro  Glu  Thr  Val  Pro  Lys
          75                        80

Pro  Cys  Cys  Ala  Pro  Thr  Gln  Leu  Asn
               85                        90

Ala  Ile  Ser  Val  Leu  Tyr  Phe  Asp  Asp
                    95

Ser  Ser  Asn  Val  Ile  Leu  Lys  Lys  Tyr
100                      105

Arg  Asn  Met  Val  Val  Arg  Ala  Cys  Gly
     110                      115

Cys  His  .
```

(2)   INFORMATION FOR SEQ ID NO:4:
      (i)     SEQUENCE CHARACTERISTICS:
              (A) LENGTH:  117 amino acids
              (B) TYPE:  amino acid
              (D) TOPOLOGY:  linear
      (ii)   MOLECULE TYPE:  protein
      (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
          Met  Ala  Asn  Val  Ala  Glu  Asn
          1                   5

Ser  Ser  Ser  Asp  Gln  Arg  Gln  Ala  Cys
          10                       15

Lys  Lys  His  Glu  Leu  Tyr  Val  Ser  Phe
               20                       25

Arg  Asp  Leu  Gly  Trp  Gln  Asp  Trp  Ile
                    30
```

-64-

```
Ile   Ala   Pro   Glu   Gly   Tyr   Ala   Ala   Tyr
35                            40

Tyr   Cys   Glu   Gly   Glu   Cys   Ala   Phe   Pro
      45                      50

Leu   Asn   Ser   Tyr   Met   Asn   Ala   Thr   Asn
            55                            60

His   Ala   Ile   Val   Gln   Thr   Leu   Val   His
                  65                            70

Phe   Ile   Asn   Pro   Glu   Thr   Val   Pro   Lys
                        75

Pro   Cys   Cys   Ala   Pro   Thr   Gln   Leu   Asn
80                            85

Ala   Ile   Ser   Val   Leu   Tyr   Phe   Asp   Asp
      90                            95

Ser   Ser   Asn   Val   Ile   Leu   Lys   Lys   Tyr
            100                           105

Arg   Asn   Met   Val   Val   Arg   Ala   Cys   Gly
                  110                           115

Cys   His .
```

(2)   INFORMATION FOR SEQ ID NO:5:

    (i)    SEQUENCE CHARACTERISTICS:

        (A) LENGTH:  116 amino acids

        (B) TYPE:  amino acid

        (D) TOPOLOGY:  linear

    (ii)   MOLECULE TYPE:  protein

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
                  Ala   Asn   Val   Ala   Glu   Asn
                  1                       5

Ser   Ser   Ser   Asp   Gln   Arg   Gln   Ala   Cys
                  10                            15

Lys   Lys   His   Glu   Leu   Tyr   Val   Ser   Phe
                        20
```

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Arg 25 | Asp | Leu | Gly | Trp | Gln 30 | Asp | Trp | Ile |
| Ile | Ala 35 | Pro | Glu | Gly | Tyr | Ala 40 | Ala | Tyr |
| Tyr | Cys | Glu 45 | Gly | Glu | Cys | Ala | Phe 50 | Pro |
| Leu | Asn | Ser | Tyr 55 | Met | Asn | Ala | Thr | Asn 60 |
| His | Ala | Ile | Val | Gln 65 | Thr | Leu | Val | His |
| Phe 70 | Ile | Asn | Pro | Glu | Thr 75 | Val | Pro | Lys |
| Pro | Cys 80 | Cys | Ala | Pro | Thr | Gln 85 | Leu | Asn |
| Ala | Ile | Ser 90 | Val | Leu | Tyr | Phe | Asp 95 | Asp |
| Ser | Ser | Asn | Val 100 | Ile | Leu | Lys | Lys | Tyr 105 |
| Arg | Asn | Met | Val | Val 110 | Arg | Ala | Cys | Gly |
| Cys 115 | His . | | | | | | | |

(2)   INFORMATION FOR SEQ ID NO:6:

    (i)     SEQUENCE CHARACTERISTICS:

        (A) LENGTH:   114 amino acids

        (B) TYPE:   amino acid

        (D) TOPOLOGY:   linear

    (ii)   MOLECULE TYPE:   protein

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:6:

Val   Ala   Glu   Asn   Ser   Ser   Ser   Asp   Gln
1                       5

Arg   Gln   Ala   Cys   Lys   Lys   His   Glu   Leu
10                      15

Tyr   Val   Ser   Phe   Arg   Asp   Leu   Gly   Trp
      20                      25

Gln   Asp   Trp   Ile   Ile   Ala   Pro   Glu   Gly
            30                      35

Tyr   Ala   Ala   Tyr   Tyr   Cys   Glu   Gly   Glu
                  40                            45

Cys   Ala   Phe   Pro   Leu   Asn   Ser   Tyr   Met
                        50

Asn   Ala   Thr   Asn   His   Ala   Ile   Val   Gln
55                            60

Thr   Leu   Val   His   Phe   Ile   Asn   Pro   Glu
      65                            70

Thr   Val   Pro   Lys   Pro   Cys   Cys   Ala   Pro
            75                            80

Thr   Gln   Leu   Asn   Ala   Ile   Ser   Val   Leu
                  85                            90

Tyr   Phe   Asp   Asp   Ser   Ser   Asn   Val   Ile
                        95

Leu   Lys   Lys   Tyr   Arg   Asn   Met   Val   Val
100                     105

Arg   Ala   Cys   Gly   Cys   His .
110

(2)   INFORMATION FOR SEQ ID NO:7:
      (i)   SEQUENCE CHARACTERISTICS:
            (A) LENGTH:   1822 base pairs
            (B) TYPE:   nucleic acid
            (C) STRANDEDNESS:   single
            (D) TOPOLOGY:   linear

(ii)   MOLECULE TYPE:  cDNA to mRNA

(iii)  HYPOTHETICAL:  no

(iv)   ANTI-SENSE:  no

(vi)   ORIGINAL SOURCE:

      (A) ORGANISM:  Bovinae

      (F) TISSUE TYPE:  bone

(vii)  IMMEDIATE SOURCE:

      (A) LIBRARY:  human placenta

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:7:


```
GGTGCGGGCC CGGAGCCCGG AGCCCGGGTA GCGCGTAGAG          40


CCGGCGCG ATG CAC GTG CGC TCA CTG CGA GCT GCG          75
         Met His Val Arg Ser Leu Arg Ala Ala
          1               5

GCG CCG CAC AGC TTC GTG GCG CTC TGG GCA CCC          108
Ala Pro His Ser Phe Val Ala Leu Trp Ala Pro
 10               15                      20

CTG TTC CTG CTG CGC TCC GCC CTG GCC GAC TTC          141
Leu Phe Leu Leu Arg Ser Ala Leu Ala Asp Phe
                 25                  30

AGC CTG GAC AAC GAG GTG CAC TCG AGC TTC ATC          174
Ser Leu Asp Asn Glu Val His Ser Ser Phe Ile
                 35                  40

CAC CGG CGC CTC CGC AGC CAG GAG CGG CGG GAG          207
His Arg Arg Leu Arg Ser Gln Glu Arg Arg Glu
             45                  50

ATG CAG CGC GAG ATC CTC TCC ATT TTG GGC TTG          240
Met Gln Arg Glu Ile Leu Ser Ile Leu Gly Leu
         55                  60

CCC CAC CGC CCG CGC CCG CAC CTC CAG GGC AAG          273
Pro His Arg Pro Arg Pro His Leu Gln Gly Lys
 65                  70                  75
```

```
CAC AAC TCG GCA CCC ATG TTC ATG CTG GAC CTG        306
His Asn Ser Ala Pro Met Phe Met Leu Asp Leu
                 80              85

TAC AAC GCC ATG GCG GTG GAG GAG GGC GGC GGG        339
Tyr Asn Ala Met Ala Val Glu Glu Gly Gly Gly
                 90              95

CCC GGC GGC CAG GGC TTC TCC TAC CCC TAC AAG        372
Pro Gly Gly Gln Gly Phe Ser Tyr Pro Tyr Lys
             100             105

GCC GTC TTC AGT ACC CAG GGC CCC CCT CTG GCC        405
Ala Val Phe Ser Thr Gln Gly Pro Pro Leu Ala
             110             115

AGC CTG CAA GAT AGC CAT TTC CTC ACC GAC GCC        438
Ser Leu Gln Asp Ser His Phe Leu Thr Asp Ala
120                 125                 130

GAC ATG GTC ATG AGC TTC GTC AAC CTC GTG GAA        471
Asp Met Val Met Ser Phe Val Asn Leu Val Glu
                 135             140

CAT GAC AAG GAA TTC TTC CAC CCA CGC TAC CAC        504
His Asp Lys Glu Phe Phe His Pro Arg Tyr His
             145             150

CAT CGA GAG TTC CGG TTT GAT CTT TCC AAG ATC        537
His Arg Glu Phe Arg Phe Asp Leu Ser Lys Ile
             155             160

CCA GAA GGG GAA GCT GTC ACG GCA GCC GAA TTC        570
Pro Glu Gly Glu Ala Val Thr Ala Ala Glu Phe
165                 170

CGG ATC TAC AAG GAC TAC ATC CGG GAA CGC TTC        603
Arg Ile Tyr Lys Asp Tyr Ile Arg Glu Arg Phe
175                 180                 185

GAC AAT GAG ACG TTC CGG ATC AGC GTT TAT CAG        636
Asp Asn Glu Thr Phe Arg Ile Ser Val Tyr Gln
                 190             195

GTG CTC CAG GAG CAC TTG GGC AGG GAA TCG GAT        669
```

```
    Val Leu Gln Glu His Leu Gly Arg Glu Ser Asp
                200                 205

CTC TTC CTG CTC GAC AGC CGT ACC CTC TGG GCC      702
Leu Phe Leu Leu Asp Ser Arg Thr Leu Trp Ala
        210                 215

TCG GAG GAG GGC TGG CTG GTG TTT GAC ATC ACA      735
Ser Glu Glu Gly Trp Leu Val Phe Asp Ile Thr
    220                 225

GCC ACC AGC AAC CAC TGG GTG GTC AAT CCG CGG      768
Ala Thr Ser Asn His Trp Val Val Asn Pro Arg
230                 235                 240

CAC AAC CTG GGC CTG CAG CTC TCG GTG GAG ACG      801
His Asn Leu Gly Leu Gln Leu Ser Val Glu Thr
                245                 250

CTG GAT GGG CAG AGC ATC AAC CCC AAG TTG GCG      834
Leu Asp Gly Gln Ser Ile Asn Pro Lys Leu Ala
                255                 260

GGC CTG ATT GGG CGG CAC GGG CCC CAG AAC AAG      867
Gly Leu Ile Gly Arg His Gly Pro Gln Asn Lys
            265                 270

CAG CCC TTC ATG GTG GCT TTC TTC AAG GCC ACG      900
Gln Pro Phe Met Val Ala Phe Phe Lys Ala Thr
    275                 280

GAG GTC CAC TTC CGC AGC ATC CGG TCC ACG GGG      933
Glu Val His Phe Arg Ser Ile Arg Ser Thr Gly
285                 290                 295

AGC AAA CAG CGC AGC CAG AAC CGC TCC AAG ACG      966
Ser Lys Gln Arg Ser Gln Asn Arg Ser Lys Thr
                300                 305

CCC AAG AAC CAG GAA GCC CTG CGG ATG GCC AAC      999
Pro Lys Asn Gln Glu Ala Leu Arg Met Ala Asn
                310                 315

GTG GCA GAG AAC AGC AGC AGC GAC CAG AGG CAG     1032
```

```
             Val Ala Glu Asn Ser Ser Ser Asp Gln Arg Gln
                     320                 325

GCC TGT AAG AAG CAC GAG CTG TAT GTC AGC TTC            1065
Ala Cys Lys Lys His Glu Leu Tyr Val Ser Phe
        330                 335

CGA GAC CTG GGC TGG CAG GAC TGG ATC ATC GCG            1098
Arg Asp Leu Gly Trp Gln Asp Trp Ile Ile Ala
340                 345                 350

CCT GAA GGC TAC GCC GCC TAC TAC TGT GAG GGG            1131
Pro Glu Gly Tyr Ala Ala Tyr Tyr Cys Glu Gly
                355                 360

GAG TGT GCC TTC CCT CTG AAC TCC TAC ATG AAC            1164
Glu Cys Ala Phe Pro Leu Asn Ser Tyr Met Asn
                365                 370

GCC ACC AAC CAC GCC ATC GTG CAG ACG CTG GTC            1197
Ala Thr Asn His Ala Ile Val Gln Thr Leu Val
        375                 380

CAC TTC ATC AAC CCG GAA ACG GTG CCC AAG CCC            1230
His Phe Ile Asn Pro Glu Thr Val Pro Lys Pro
        385                 390

TGC TGT GCG CCC ACG CAG CTC AAT GCC ATC TCC            1263
Cys Cys Ala Pro Thr Gln Leu Asn Ala Ile Ser
395                 400                 405

GTC CTC TAC TTC GAT GAC AGC TCC AAC GTC ATC            1296
Val Leu Tyr Phe Asp Asp Ser Ser Asn Val Ile
                410                 415

CTG AAG AAA TAC AGA AAC ATG GTG GTC CGG GCC            1329
Leu Lys Lys Tyr Arg Asn Met Val Val Arg Ala
                420                 425

TGT GGC TGC CAC TAGCTCCTCC GAGAATTCAG                  1361
Cys Gly Cys His
                430

ACCCTTTGGG GCCAAGTTTT TCTGGATCCT CCATTGCTCG            1401
```

CCTTGGCCAG GAACCAGCAG ACCAACTGCC TTTTGTGAGA     1441

CCTTCCCCTC CCTATCCCCA ACTTTAAAGG TGTGAGAGTA     1481

TTAGGAAACA TGAGCAGCAT ATGGCTTTTG ATCAGTTTTT     1521

CAGTGGCAGC ATCCAATGAA CAAGATCCTA CAAGCTGTGC     1561

AGGCAAAACC TAGCAGGAAA AAAAAACAAC GCATAAAGAA     1601

AAATGGCCGG GCCAGGTCAT TGGCTGGGAA GTCTCAGCCA     1641

TGCACGGACT CGTTTCCAGA GGTAATTATG AGCGCCTACC     1681

AGCCAGGCCA CCCAGCCGTG GGAGGAAGGG GGCGTGGCAA     1721

GGGGTGGGCA CATTGGTGTC TGTGCGAAAG GAAAATTGAC     1761

CCGGAAGTTC CTGTAATAAA TGTCACAATA AAACGAATGA     1801

ATGAAAAAAA AAAAAAAAA A     1822

What is claimed is:


1.       An osteogenic device for implantation in a mammal, the device comprising:

a biocompatible, in vivo biodegradable matrix of mineral-free, delipidated Type I insoluble bone collagen particles, depleted in noncollagenous protein; and

a protein produced by the expression of recombinant DNA in a mammalian host cell, the protein comprising two oxidized subunits, the amino acid sequence of each subunit being sufficiently duplicative of the amino sequence (Seq. ID No.6):


<u>OP1-16V</u>

```
                                                        30
                                     V   A   E   N   S
                                 40
    S   S   D   Q   R   Q   A   C   K   K   H   E   L   Y   V
                50                                          60
    S   F   R   D   L   G   W   Q   D   W   I   I   A   P   E
                                     70
    G   Y   A   A   Y   Y   C   E   G   E   C   A   F   P   L
                80                                          90
    N   S   Y   M   N   A   T   N   H   A   I   V   Q   T   L
                                    100
    V   H   F   I   N   P   E   T   V   P   K   P   C   C   A
               110                                         120
    P   T   Q   L   N   A   I   S   V   L   Y   F   D   D   S
                                    130
    S   N   V   I   L   K   K   Y   R   N   M   V   V   R   A

    C   G   C   H,
```


such that the dimeric species comprising said subunits has a conformation that is capable of inducing endochondral bone formation in a mammal when disposed within said matrix and implanted in said mammal.

2.        An osteogenic protein expressed from
recombinant DNA in a mammalian host cell and capable
of inducing endochondral bone formation in a mammal
when disposed within a matrix implanted in said
mammal;

        a protein produced by the expression of
recombinant DNA in a mammalian host cell, the protein
comprising two oxidized subunits, the amino acid
sequence of each subunit being sufficiently
duplicative of the amino sequence (Seq. ID No.6):

<u>OP1-16V</u>

```
                                                    30
                                      V   A   E   N   S
                                  40
    S   S   D   Q   R   Q   A   C   K   K   H   E   L   Y   V
                      50                                  60
    S   F   R   D   L   G   W   Q   D   W   I   I   A   P   E
                                  70
    G   Y   A.  A   Y   Y   C   E   G   E   C   A   F   P   L
                  80                                      90
    N   S   Y   M   N   A   T   N   H   A   I   V   Q   T   L
                                 100
    V   H   F   I   N   P   E   T   V   P   K   P   C   C   A
                 110                                     120
    P   T   Q   L   N   A   I   S   V   L   Y   F   D   D   S
                                 130
    S   N   V   I   L   K   K   Y   R   N   M   V   V   R   A

    C   G   C   H,
```

        such that the dimeric species comprising
said subunits has a conformation that is capable of
inducing endochondral bone formation in a mammal when
disposed within said matrix and implanted in said
mammal.

3.      The invention of claim 1 or 2 wherein the
amino acid sequence of each said subunit has at least
70% homology with the amino acid sequence (Seq. ID
No.6):


OP1-16V


```
                                         30
                        V   A   E   N   S
                                40
S   S   D   Q   R   Q   A   C   K   K   H   E   L   Y   V
                50                              60
S   F   R   D   L   G   W   Q   D   W   I   I   A   P   E
                                70
G   Y   A   A   Y   Y   C   E   G   E   C   A   F   P   L
                80                              90
N   S   Y   M   N   A   T   N   H   A   I   V   Q   T   L
                                100
V   H   F   I   N   P   E   T   V   P   K   P   C   C   A
                110                             120
P   T   Q   L   N   A   I   S   V   L   Y   F   D   D   S
                                130
S   N   V   I   L   K   K   Y   R   N   M   V   V   R   A

C   G   C   H.
```


4.      The invention of claim 1 or 2 wherein
the amino acid sequence of each said subunit has
at least 80% homology with the amino acid
sequence (Seq. ID No.6):


OP1-16V


```
                                         30
                        V   A   E   N   S
                                40
S   S   D   Q   R   Q   A   C   K   K   H   E   L   Y   V
                50                              60
S   F   R   D   L   G   W   Q   D   W   I   I   A   P   E
                                70
G   Y   A   A   Y   Y   C   E   G   E   C   A   F   P   L
```

```
                        80                                    90
     N    S    Y    M    N    A    T    N    H    A    I    V    Q    T    L
                                            100
     V    H    F    I    N    P    E    T    V    P    K    P    C    C    A
                   110                                           120
     P    T    Q    L    N    A    I    S    V    L    Y    F    D    D    S
                                       130
     S    N    V    I    L    K    K    Y    R    N    M    V    V    R    A

     C    G    C    H.
```

5.     The invention of claim 1 or 2 wherein the amino acid sequence of each said subunit comprises (Seq. ID No. 6):


<u>OP1-16V</u>

```
                                                                  30
                                            V    A    E    N    S
                                       40
     S    S    D    Q    R    Q    A    C    K    K    H    E    L    Y    V
                   50                                            60
     S    F    R    D    L    G    W    Q    D    W    I    I    A    P    E
                                            70
     G    Y    A    A    Y    Y    C    E    G    E    C    A    F    P    L
                        80                                    90
     N    S    Y    M    N    A    T    N    H    A    I    V    Q    T    L
                                            100
     V    H    F    I    N    P    E    T    V    P    K    P    C    C    A
                   110                                           120
     P    T    Q    L    N    A    I    S    V    L    Y    F    D    D    S
                                       130
     S    N    V    I    L    K    K    Y    R    N    M    V    V    R    A

     C    G    C    H.
```

6.     The invention of claim 1 or 2 wherein the amino acid sequence of said subunit comprises (Seq. ID No.1):

## OP1-18

```
1                                    10
S   T   G   S   K   Q   R   S   Q   N   R   S   K   T   P
            20                                      30
K   N   Q   E   A   L   R   M   A   N   V   A   E   N   S
                            40
S   S   D   Q   R   Q   A   C   K   K   H   E   L   Y   V
            50                                      60
S   F   R   D   L   G   W   Q   D   W   I   I   A   P   E
                            70
G   Y   A   A   Y   Y   C   E   G   E   C   A   F   P   L
            80                                      90
N   S   Y   M   N   A   T   N   H   A   I   V   Q   T   L
                           100
V   H   F   I   N   P   E   T   V   P   K   P   C   C   A
            110                                    120
P   T   Q   L   N   A   I   S   V   L   Y   F   D   D   S
                           130
S   N   V   I   L   K   K   Y   R   N   M   V   V   R   A

C   G   C   H.
```

7.      The invention of claim 1 or 2 wherein
the amino acid sequence of each said subunit
comprises (Seq. ID No. 2):

## OP1-16S

```
                            8       10
                            S   Q   N   R   S   K   T   P
            20                                      30
K   N   Q   E   A   L   R   M   A   N   V   A   E   N   S
                            40
S   S   D   Q   R   Q   A   C   K   K   H   E   L   Y   V
            50                                      60
S   F   R   D   L   G   W   Q   D   W   I   I   A   P   E
                            70
G   Y   A   A   Y   Y   C   E   G   E   C   A   F   P   L
            80                                      90
N   S   Y   M   N   A   T   N   H   A   I   V   Q   T   L
                           100
V   H   F   I   N   P   E   T   V   P   K   P   C   C   A
            110                                    120
P   T   Q   L   N   A   I   S   V   L   Y   F   D   D   S
                           130
S   N   V   I   L   K   K   Y   R   N   M   V   V   R   A

C   G   C   H.
```

8.      The invention of claim 1 or 2 wherein
the amino acid sequence of each said subunit
comprises (Seq. ID No. 3):


### OP1-16L

```
              21                          30
              L  R  M  A  N  V  A  E  N  S
                          40
    S  S  D  Q  R  Q  A  C  K  K  H  E  L  Y  V
                50                          60
    S  F  R  D  L  G  W  Q  D  W  I  I  A  P  E
                          70
    G  Y  A  A  Y  Y  C  E  G  E  C  A  F  P  L
                80                          90
    N  S  Y  M  N  A  T  N  H  A  I  V  Q  T  L
                          100
    V  H  F  I  N  P  E  T  V  P  K  P  C  C  A
                110                        120
    P  T  Q  L  N  A  I  S  V  L  Y  F  D  D  S
                          130
    S  N  V  I  L  K  K  Y  R  N  M  V  V  R  A

    C  G  C  H.
```


9.      The invention of claim 1 or 2 wherein
the amino acid sequence of each of said subunit
comprises (Seq. ID No. 4):


### OP1-16M

```
              23                          30
              M  A  N  V  A  E  N  S
                       40
    S  S  D  Q  R  Q  A  C  K  K  H  E  L  Y  V
                50                          60
    S  F  R  D  L  G  W  Q  D  W  I  I  A  P  E
                          70
    G  Y  A  A  Y  Y  C  E  G  E  C  A  F  P  L
                80                          90
    N  S  Y  M  N  A  T  N  H  A  I  V  Q  T  L
                          100
    V  H  F  I  N  P  E  T  V  P  K  P  C  C  A
```

```
              110                              120
P   T   Q   L   N   A   I   S   V   L   Y   F   D   D   S
                                130
S   N   V   I   L   K   K   Y   R   N   M   V   V   R   A

C   G   C   H.
```

10.      The invention of claim 1 or 2 wherein the amino acid sequence of each of said subunit comprises (Seq. ID No. 5):

<div align="center">

OP1-16A

</div>

```
                              24                          30
                              A   N   V   A   E   N   S
                                    40
S   S   D   Q   R   Q   A   C   K   K   H   E   L   Y   V
          50                                          60
S   F   R   D   L   G   W   Q   D   W   I   I   A   P   E
                                    70
G   Y   A   A   Y   Y   C   E   G   E   C   A   F   P   L
                  80                                  90
N   S   Y   M   N   A   T   N   H   A   I   V   Q   T   L
                                   100
V   H   F   I   N   P   E   T   V   P   K   P   C   C   A
                  110                                 120
P   T   Q   L   N   A   I   S   V   L   Y   F   D   D   S
                                   130
S   N   V   I   L   K   K   Y   R   N   M   V   V   R   A

C   G   C   H.
```

11.      The invention of claim 1 or 2 wherein said protein has an apparent molecular weight of about 30 kD when oxidized, as determined by comparison to molecular weight standards in SDS-polyacrylamide gel electrophoresis.

12.      The invention of claim 1 or 2 wherein said protein has an apparent molecular weight of about 36 kD when oxidized, as determined by comparison to molecular weight standards in SDS-polyacrylamide gel electrophoresis.

13.       The invention of claim 1 or 2 wherein said protein is unglycosylated.

14.       The invention of claim 1 or 2 wherein said mammalian host cell is a chinese hamster ovary cell.

15.       The invention of claim 1 or 2 wherein said mammalian host cell is a simian kidney cell.

16.       A biocompatible, in vivo biodegradable matrix for implantation in a mammal comprising demineralized, delipidated, Type I insoluble bone collagen particles, depleted in noncollagenous protein, and treated with a hot aqueous medium having a temperature above about 37°C in an amount and for a time sufficient to alter the morphology of said particles.

17.       The invention of claim 1 or 16 wherein said matrix is treated with a hot aqueous medium having a temperature within the range of 37°C to 65°C.

18.       The invention of claim 1 or 16 wherein said matrix is treated with a hot aqueous medium having a temperature within the range of 45°C to 60°C.

19.       The invention of claim 1 or 16 wherein said matrix is treated to increase the number of pores and micropits on said collagen particles at least 3-fold.

20.    The invention of claim 1 or 16 wherein
said matrix is treated to increase the number of
pores and micropits on said collagen particles
at least 10-fold.

21.    The invention of claim 1 or 16 wherein
said bone collagen particles comprise pores or
micropits having a mean diameter within the
range of 1μm to 100μm.

22.    The invention of claim 1 or 16 wherein
said collagen particles have a mean diameter
within the range of 70 mm to 420 mm.

23.    Osteogenic protein expressed from
recombinant DNA in a mammalian host cell, said
protein comprising two oxidized subunits
constituting a dimeric species, the amino acid
sequence of said subunits having sufficient
homology with the amino acid sequence encoded by
the gene of Figure 2 (Seq. ID No. 7) such that
said dimeric species is capable of inducing bone
or cartilage formation when implanted in a
mammal in association with a matrix.

24.    A biocompatible, in vivo biodegradable
matrix for implantation in a mammal comprising
demineralized, delipidated, Type-I insoluble
bone collagen particles, depleted in a material
comprising fraction 12 identified in Figure 8.
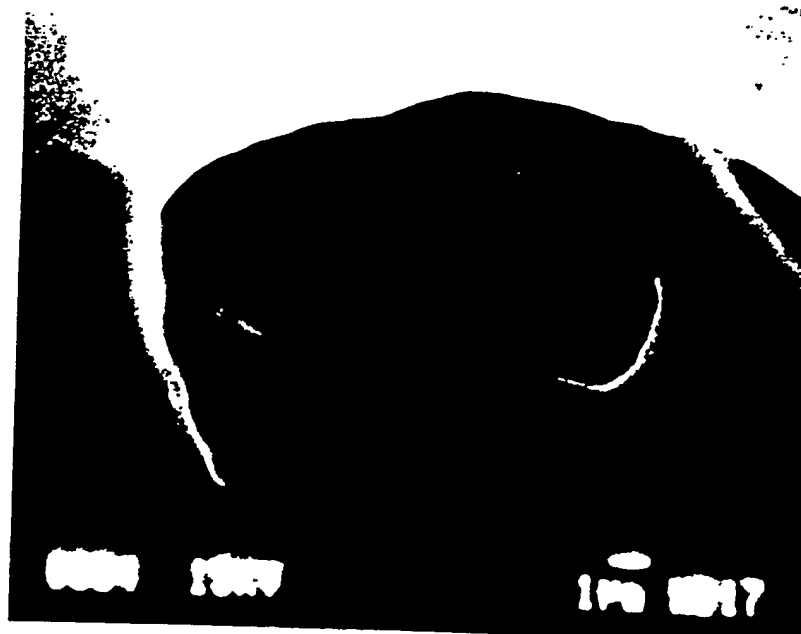
**FIG. 1A**
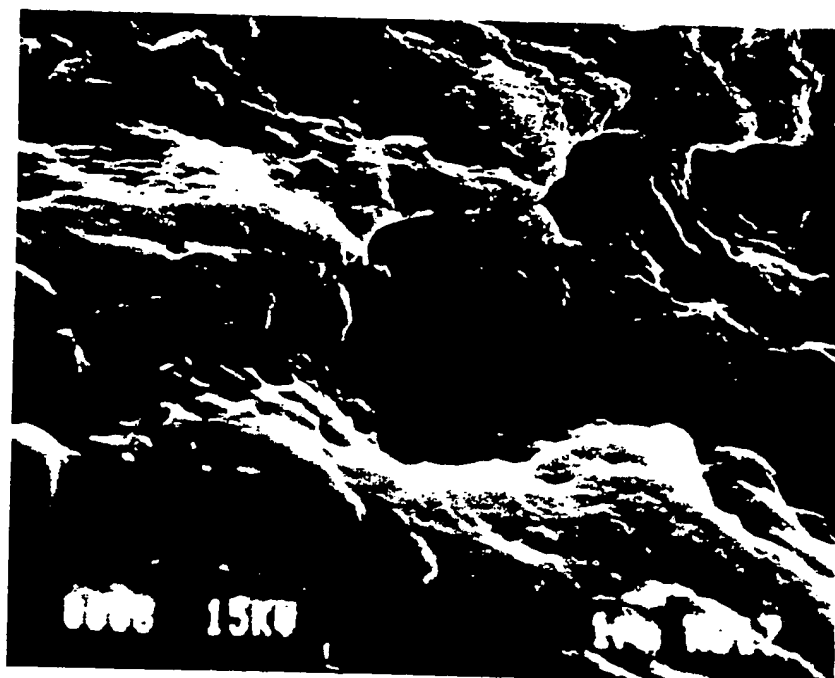


**FIG. 1B**

```
            10        20        30        40        50        60
GGTGCGGGCCCGGAGCCCGGAGCCCGGGTAGCGCGTAGAGCCGGCGCGATGCACGTGCGC
                              SmaI                          M  H  V  R
            70        80        90       100       110       120
TCACTGCGAGCTGCGGCGCCGCACAGCTTCGTGGCGCTCTGGGCACCCCTGTTCCTGCTG
    S  L  R  A  A  A  P  H  S  F  V  A  L  W  A  P  L  F  L  L
           130       140       150       160       170       180
CGCTCCGCCCTGGCCGACTTCAGCCTGGACAACGAGGTGCACTCGAGCTTCATCCACCGG
    R  S  A  L  A  D  F  S  L  D  N  E  V  H  S  S  F  I  H  R

           190       200       210       220       230       240
CGCCTCCGCAGCCAGGAGCGGCGGGAGATGCAGCGCGAGATCCTCTCCATTTTGGGCTTG
    R  L  R  S  Q  E  R  R  E  M  Q  R  E  I  L  S  I  L  G  L
           250       260       270       280       290       300
CCCCACCGCCCGCGCCCGCACCTCCAGGGCAAGCACAACTCGGCACCCATGTTCATGCTG
    P  H  R  P  R  P  H  L  Q  G  K  H  N  S  A  P  M  F  M  L
           310       320       330       340       350       360
GACCTGTACAACGCCATGGCGGTGGAGGAGGGCGGCGGGCCCGGCGGCCAGGGCTTCTCC
    D  L  Y  N  A  M  A  V  E  E  G  G  G  P  G  G  Q  G  F  S
           370       380       390       400       410       420
TACCCCTACAAGGCCGTCTTCAGTACCCAGGGCCCCCCTCTGGCCAGCCTGCAAGATAGC
    Y  P  Y  K  A  V  F  S  T  Q  G  P  P  L  A  S  L  Q  D  S
           430       440       450       460       470       480
CATTTCCTCACCGACGCCGACATGGTCATGAGCTTCGTCAACCTCGTGGAACATGACAAG
    H  F  L  T  D  A  D  M  V  M  S  F  V  N  L  V  E  H  D  K
           490       500       510       520       530       540
GAATTCTTCCACCCACGCTACCACCATCGAGAGTTCCGGTTTGATCTTTCCAAGATCCCA
    E  F  F  H  P  R  Y  H  H  R  E  F  R  F  D  L  S  K  I  P
           550       560       570       580       590       600
GAAGGGGAAGCTGTCACGGCAGCCGAATTCCGGATCTACAAGGACTACATCCGGGAACGC
    E  G  E  A  V  T  A  A  E  F  R  I  Y  K  D  Y  I  R  E  R
           610       620       630       640       650       660
TTCGACAATGAGACGTTCCGGATCAGCGTTTATCAGGTGCTCCAGGAGCACTTGGGCAGG
    F  D  N  E  T  F  R  I  S  V  Y  Q  V  L  Q  E  H  L  G  R
           670       680       690       700       710       720
GAATCGGATCTCTTCCTGCTCGACAGCCGTACCCTCTGGGCCTCGGAGGAGGGCTGGCTG
    E  S  D  L  F  L  L  D  S  R  T  L  W  A  S  E  E  G  W  L
           730       740       750       760       770       780
GTGTTTGACATCACAGCCACCAGCAACCACTGGGTGGTCAATCCGCGGCACAACCTGGGC
    V  F  D  I  T  A  T  S  N  H  W  V  V  N  P  R  H  N  L  G
           790       800       810       820       830       840
CTGCAGCTCTCGGTGGAGACGCTGGATGGGCAGAGCATCAACCCCAAGTTGGCGGGCCTG
    L  Q  L  S  V  E  T  L  D  G  Q  S  I  N  P  K  L  A  G  L
           850       860       870       880       890       900
ATTGGGCGGCACGGGCCCCAGAACAAGCAGCCCTTCATGGTGGCTTTCTTCAAGGCCACG
    I  G  R  H  G  P  Q  N  K  Q  P  F  M  V  A  F  F  K  A  T
           910       920       930       940       950       960
GAGGTCCACTTCCGCAGCATCCGGTCCACGGGGAGCAAACAGCGCAGCCAGAACCGCTCC
    E  V  H  F  R  S  I  R  S  T  G  S  K  Q  R  S  Q  N  R  S
                                   └───────>
           970       980       990      1000      1010      1020
AAGACGCCCAAGAACCAGGAAGCCCTGCGGATGGCCAACGTGGCAGAGAACAGCAGCAGC
    K  T  P  K  N  Q  E  A  L  R  M  A  N  V  A  E  N  S  S  S
          1030      1040      1050      1060      1070      1080
```

# *FIG. 2-1*

```
GACCAGAGGCAGGCCTGTAAGAAGCACGAGCTGTATGTCAGCTTCCGAGACCTGGGCTGG
 D  Q  R  Q  A  C  K  K  H  E  L  Y  V  S  F  R  D  L  G  W
    1090       1100       1110       1120       1130       1140
CAGGACTGGATCATCGCGCCTGAAGGCTACGCCGCCTACTACTGTGAGGGGGAGTGTGCC
 Q  D  W  I  I  A  P  E  G  Y  A  A  Y  Y  C  E  G  E  C  A
    1150       1160       1170       1180       1190       1200
TTCCCTCTGAACTCCTACATGAACGCCACCAACCACGCCATCGTGCAGACGCTGGTCCAC
 F  P  L  N  S  Y  M  N  A  T  N  H  A  I  V  Q  T  L  V  H
    1210       1220       1230       1240       1250       1260
TTCATCAACCCGGAAACGGTGCCCAAGCCCTGCTGTGCGCCCACGCAGCTCAATGCCATC
 F  I  N  P  E  T  V  P  K  P  C  C  A  P  T  Q  L  N  A  I
    1270       1280       1290       1300       1310       1320
TCCGTCCTCTACTTCGATGACAGCTCCAACGTCATCCTGAAGAAATACAGAAACATGGTG
 S  V  L  Y  F  D  D  S  S  N  V  I  L  K  K  Y  R  N  M  V
    1330       1340       1350       1360       1370       1380
GTCCGGGCCTGTGGCTGCCACTAGCTCCTCCGAGAATTCAGACCCTTTGGGGCCAAGTTT
 V  R  A  C  G  C  H  .*
    1390       1400       1410       1420       1430       1440
TTCTGGATCCTCCATTGCTCGCCTTGGCCAGGAACCAGCAGACCAACTGCCTTTTGTGAG
    BamH1
    1450       1460       1470       1480       1490       1500
ACCTTCCCCTCCCTATCCCCAACTTTAAAGGTGTGAGAGTATTAGGAAACATGAGCAGCA
    1510       1520       1530       1540       1550       1560
TATGGCTTTTGATCAGTTTTTCAGTGGCAGCATCCAATGAACAAGATCCTACAAGCTGTG
    1570       1580       1590       1600       1610       1620
CAGGCAAAACCTAGCAGGAAAAAAAAACAACGCATAAAGAAAAATGGCCGGGCCAGGTCA
    1630       1640       1650       1660       1670       1680
TTGGCTGGGAAGTCTCAGCCATGCACGGACTCGTTTCCAGAGGTAATTATGAGCGCCTAC
    1690       1700       1710       1720       1730       1740
CAGCCAGGCCACCCAGCCGTGGGAGGAAGGGGGCGTGGCAAGGGGTGGGCACATTGGTGT
    1750       1760       1770       1780       1790       1800
CTGTGCGAAAGGAAAATTGACCCGGAAGTTCCTGTAATAAATGTCACAATAAAACGAATG
    1810       1820
AATGAAAAAAAAAAAAAAAAAA
```

# *FIG. 2-2*
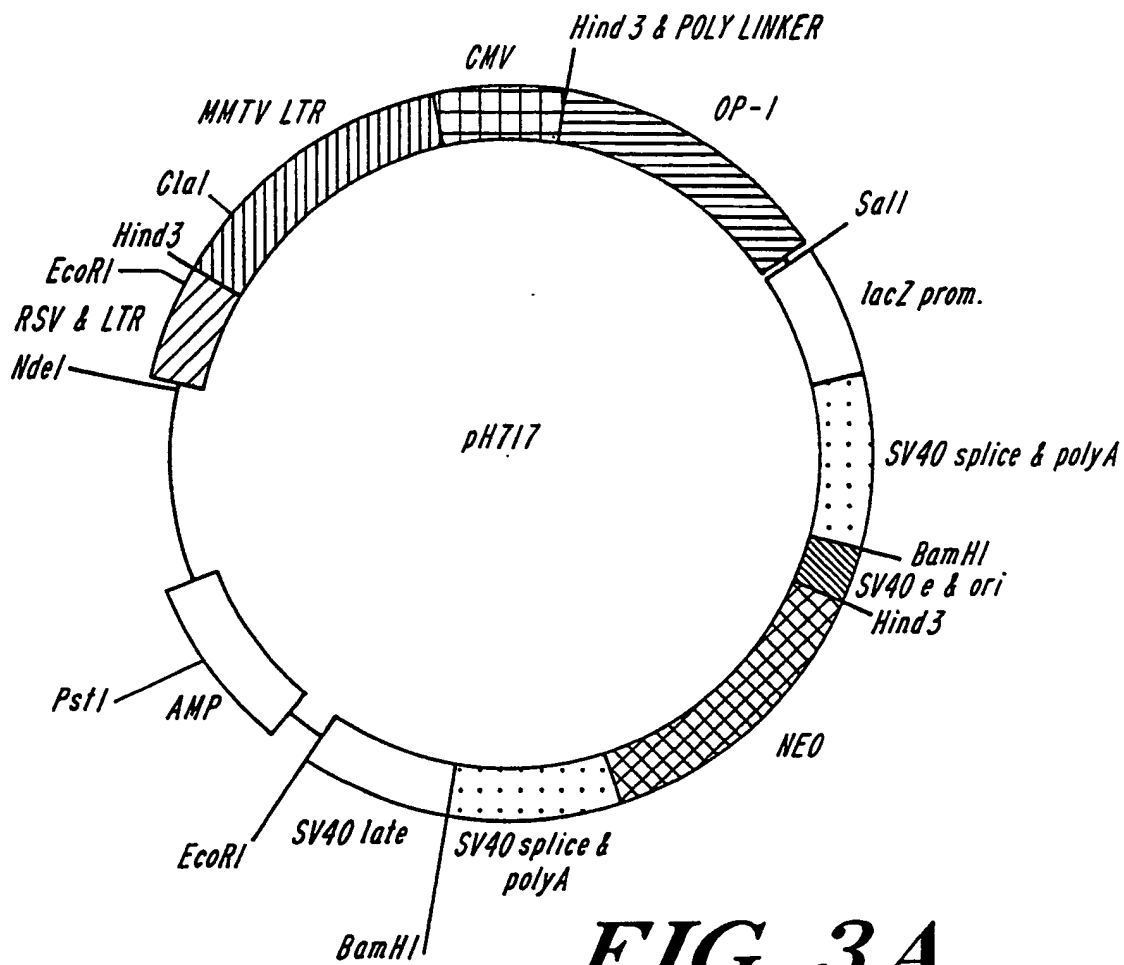
FIG. 3A
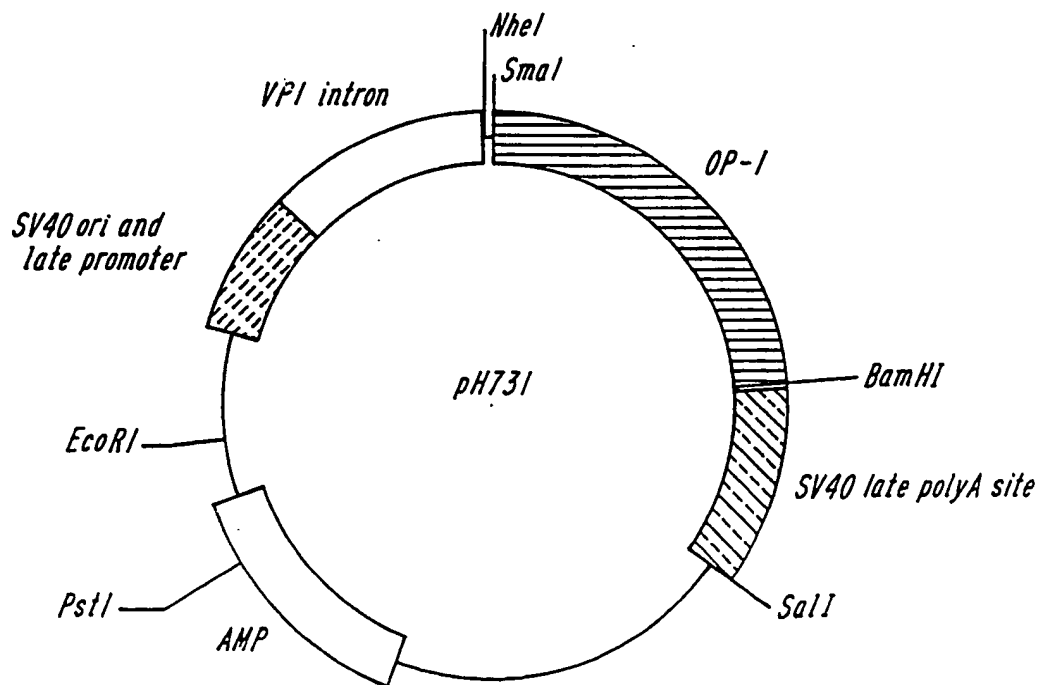


FIG. 3B
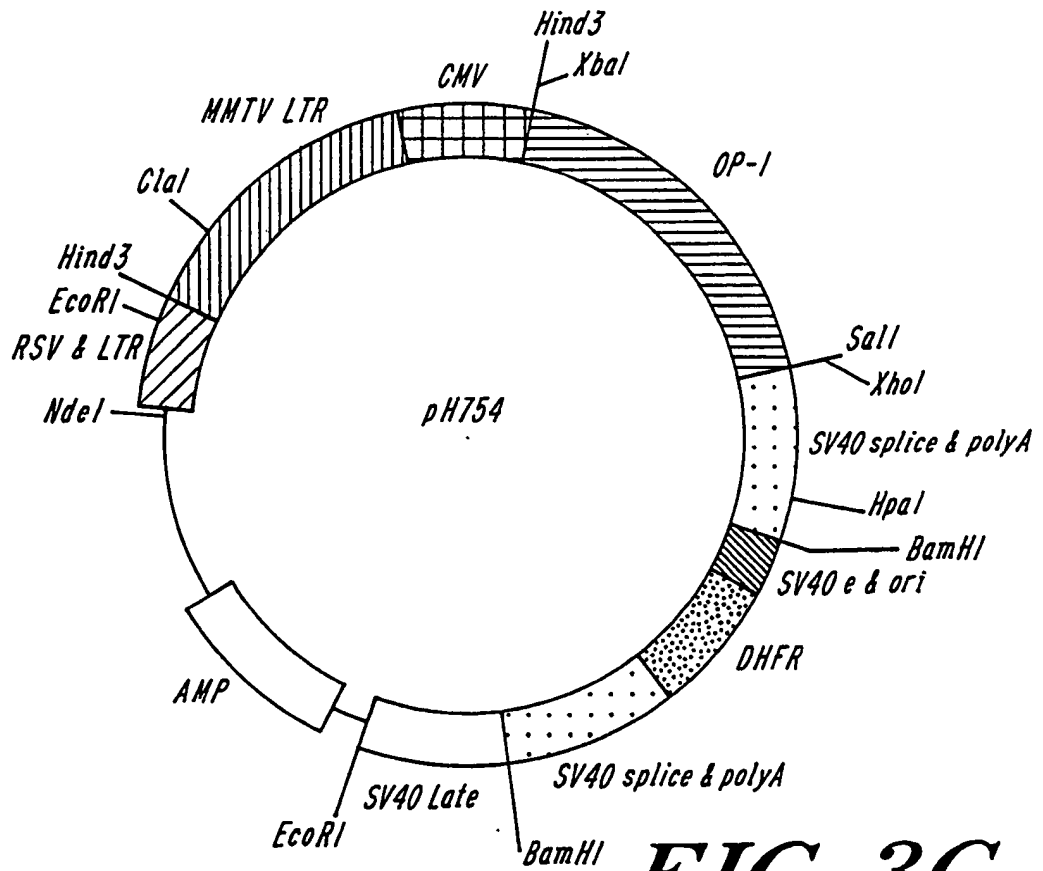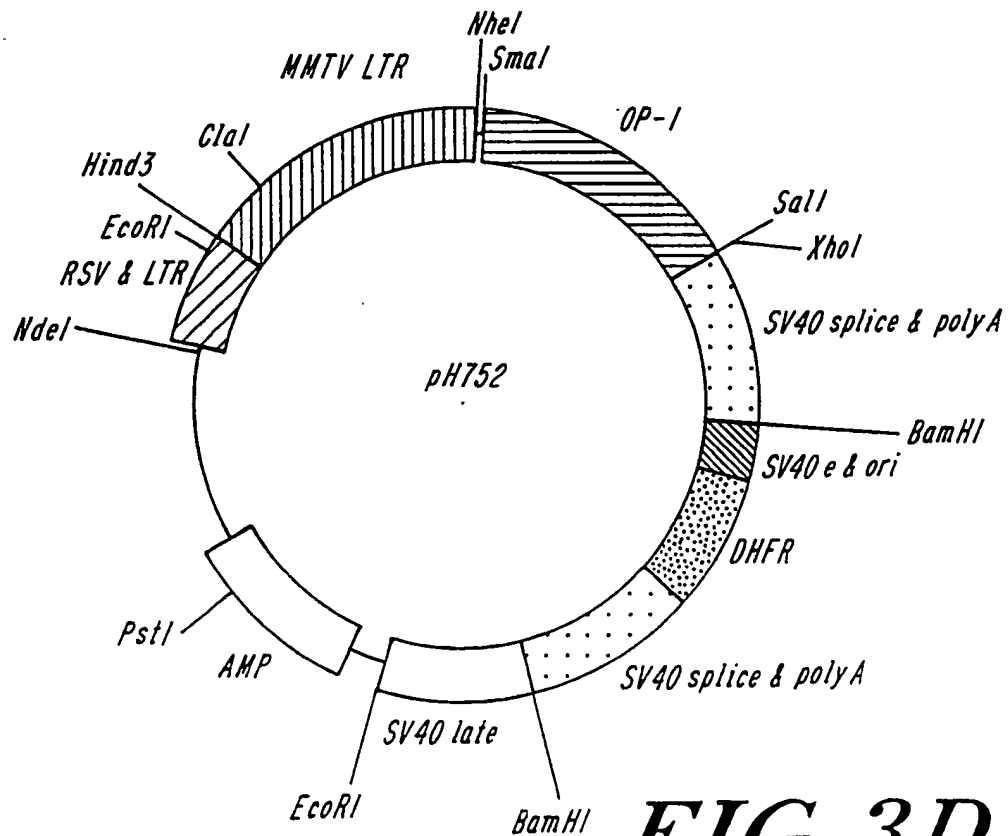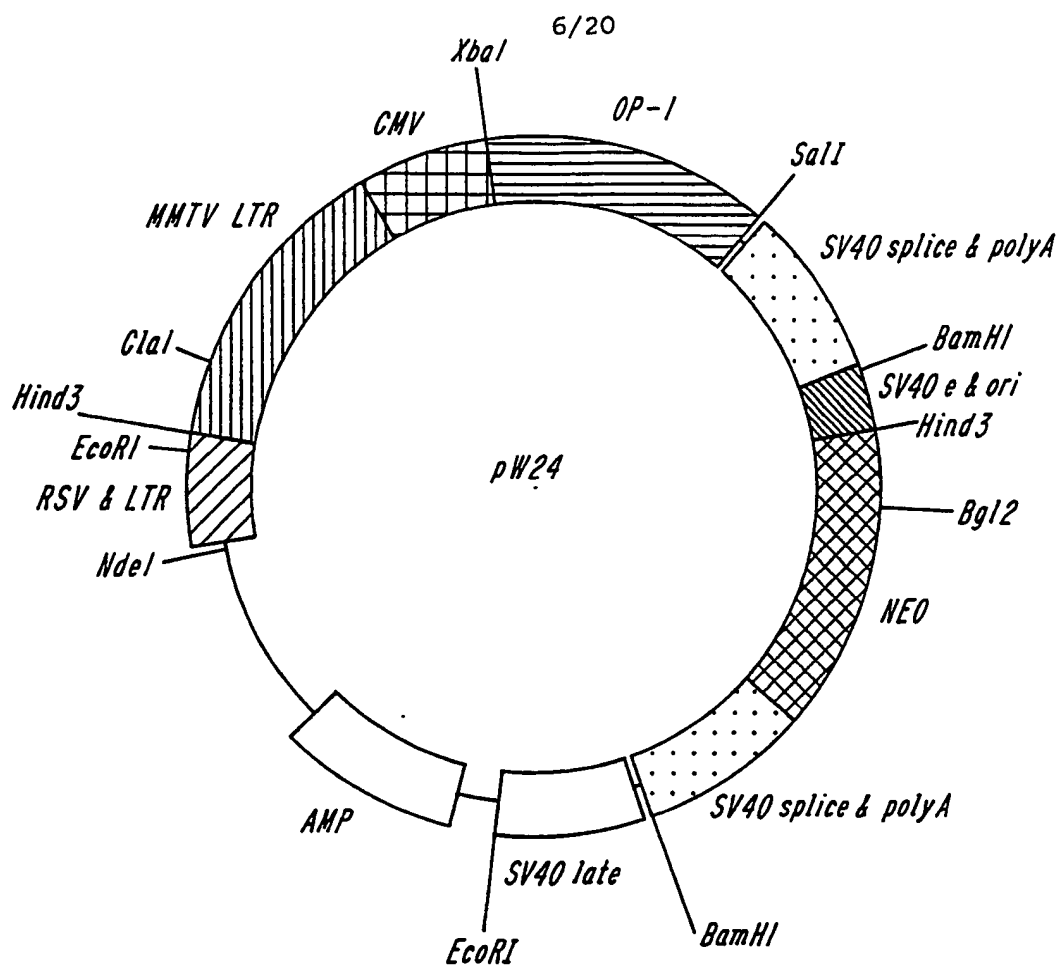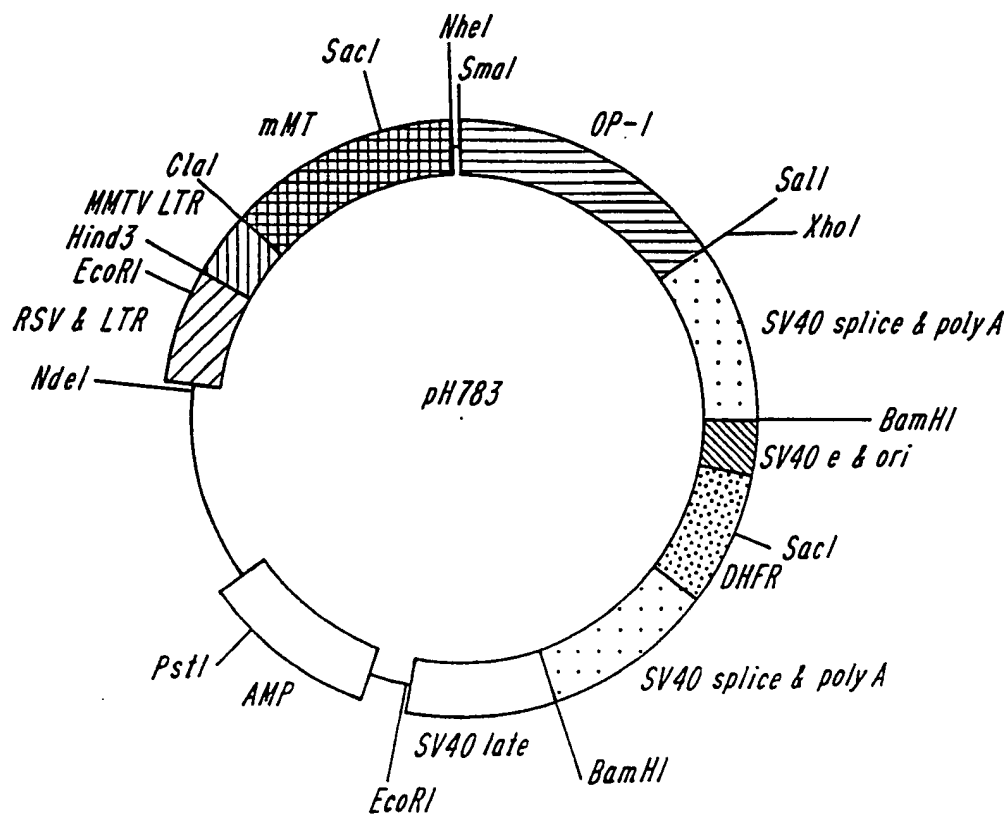
*FIG. 3C*



*FIG. 3D*

*FIG. 3E*



*FIG. 3F*

**FIG. 4**

FIG. 5A-I



FIG. 5B-I



FIG. 5C-I

uB E₁ E₂                    uB E₁ E₂
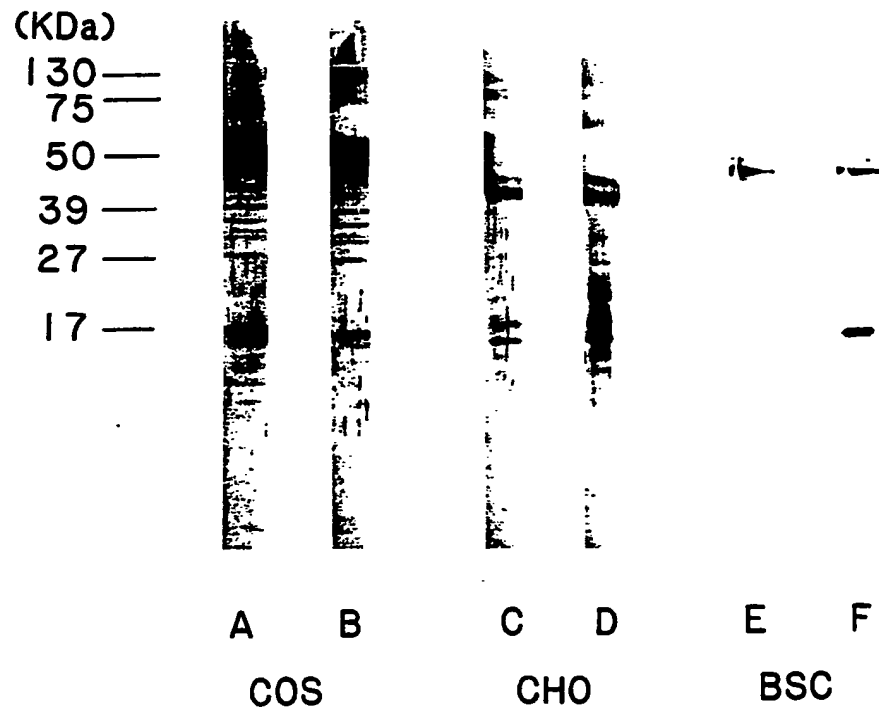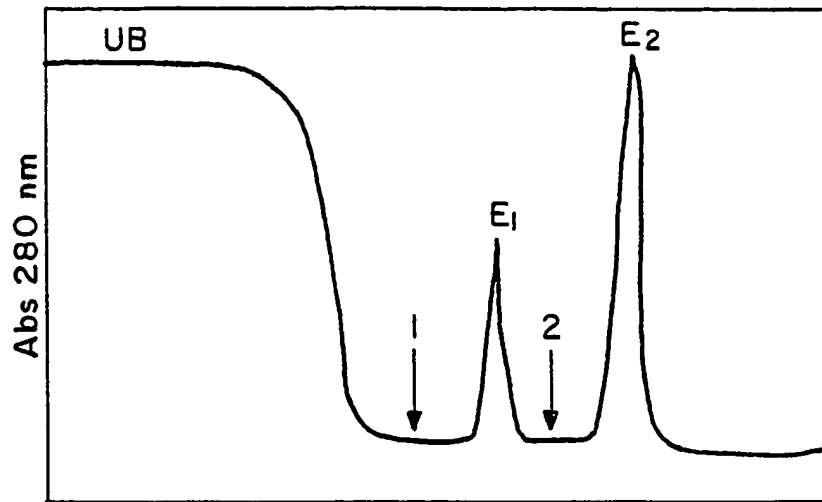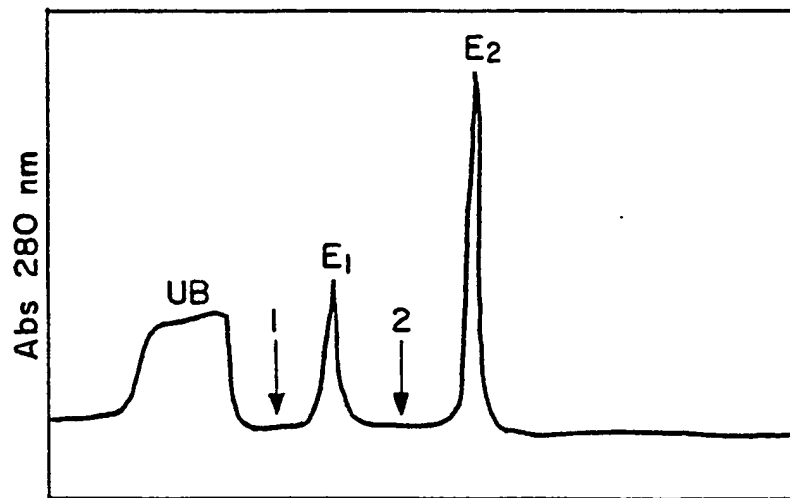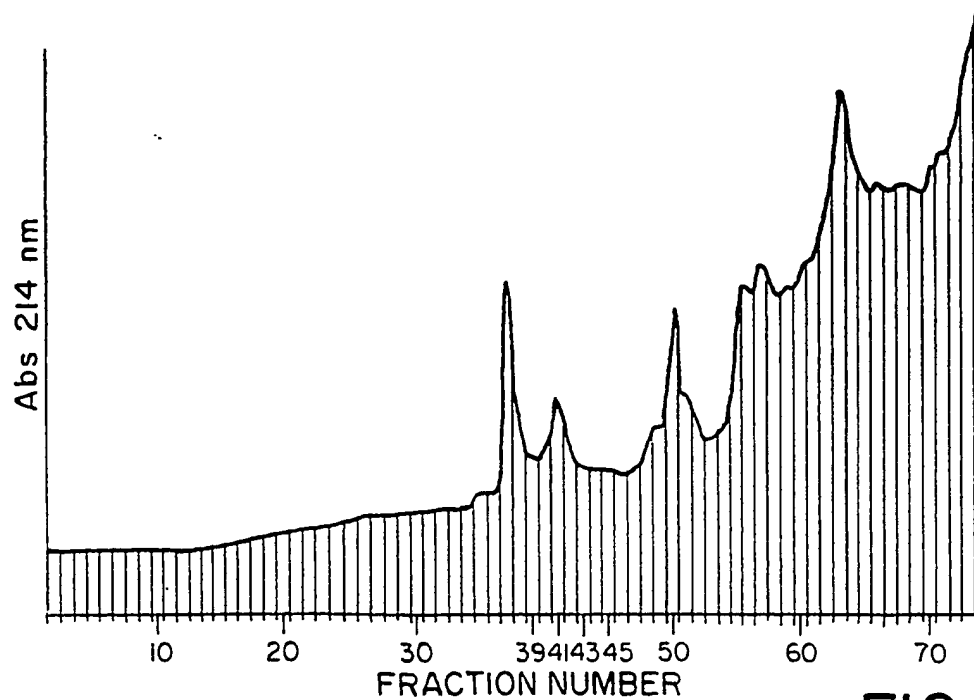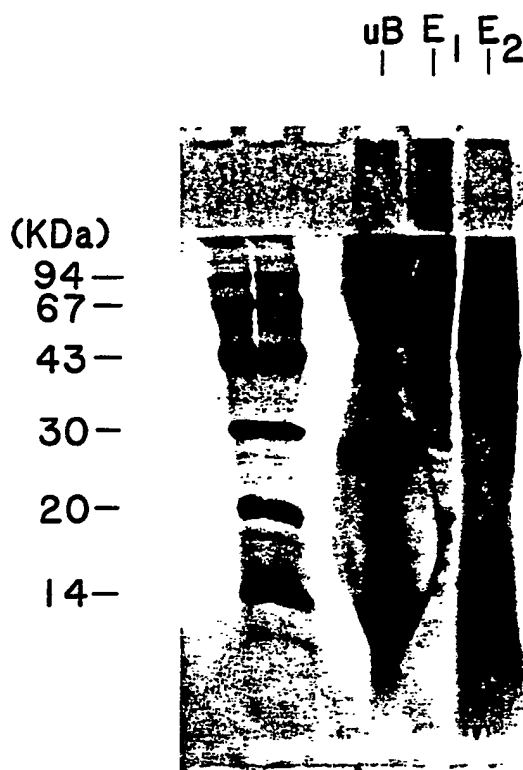


FIG. 5A-2          FIG. 5B-2
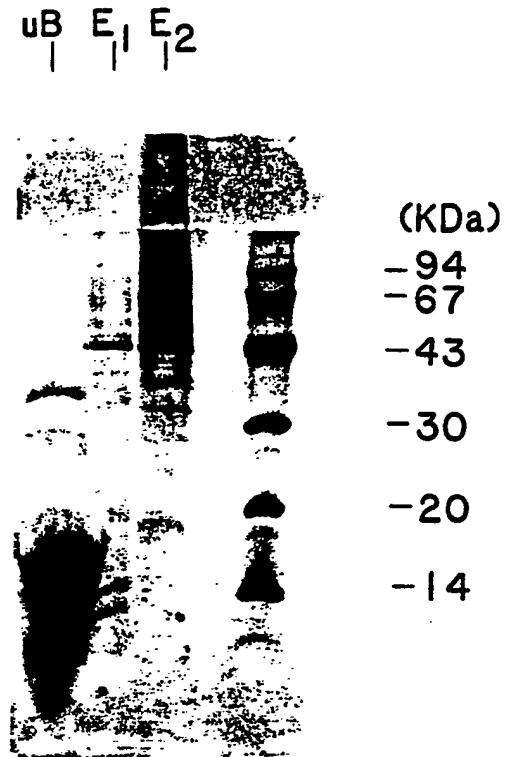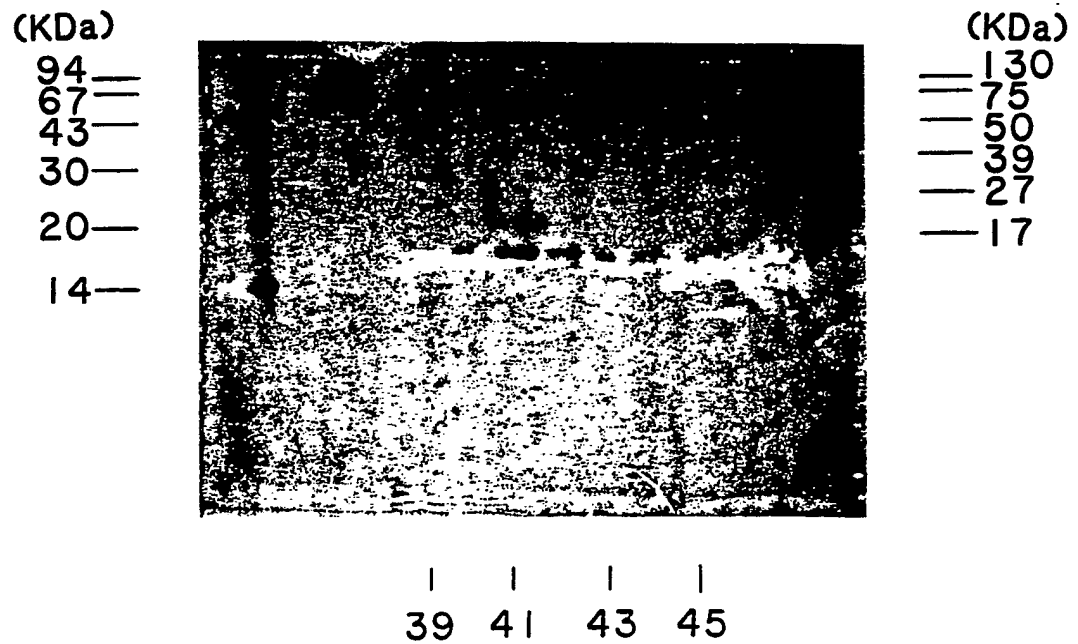


|   |   |   |   |
39  41   43  45
FRACTION NUMBER

FIG. 5C-2
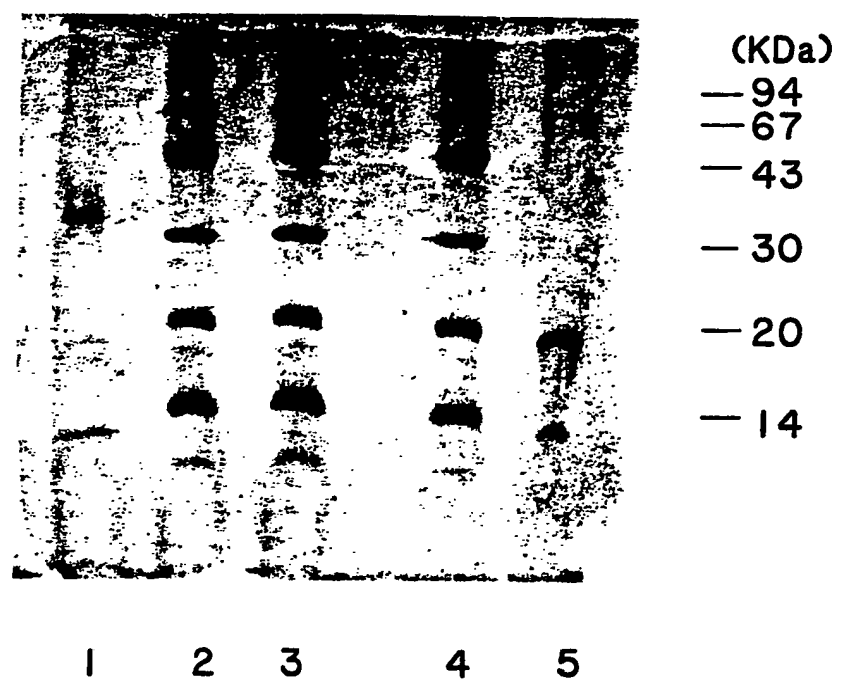
FIG. 6
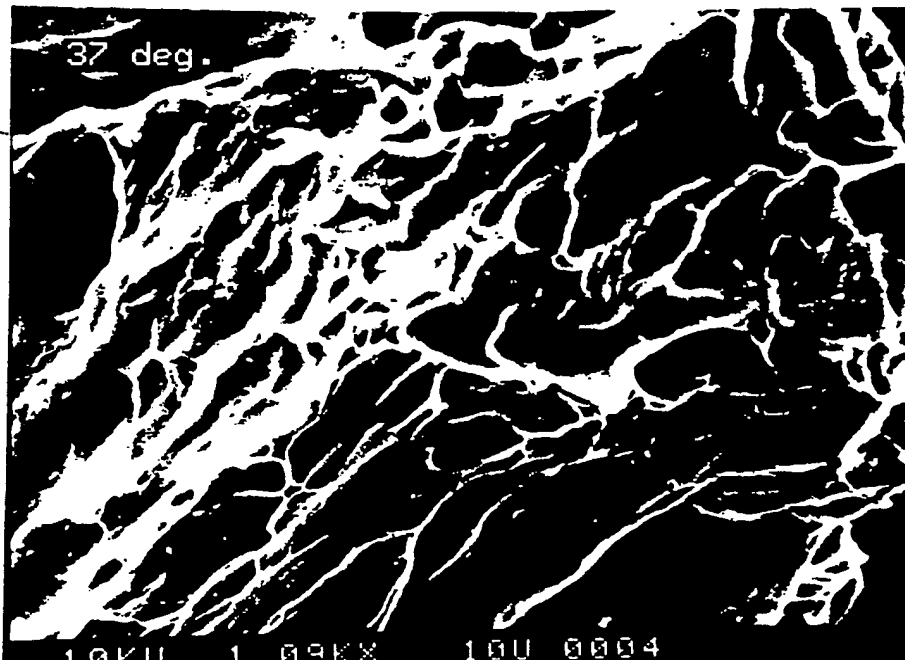
**FIG. 7A**
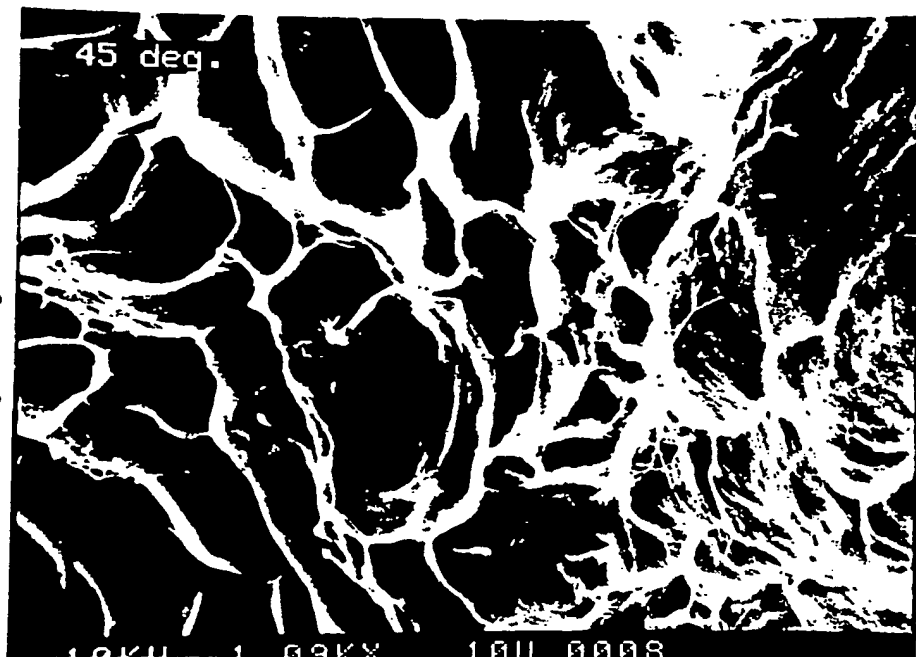


**FIG. 7B**
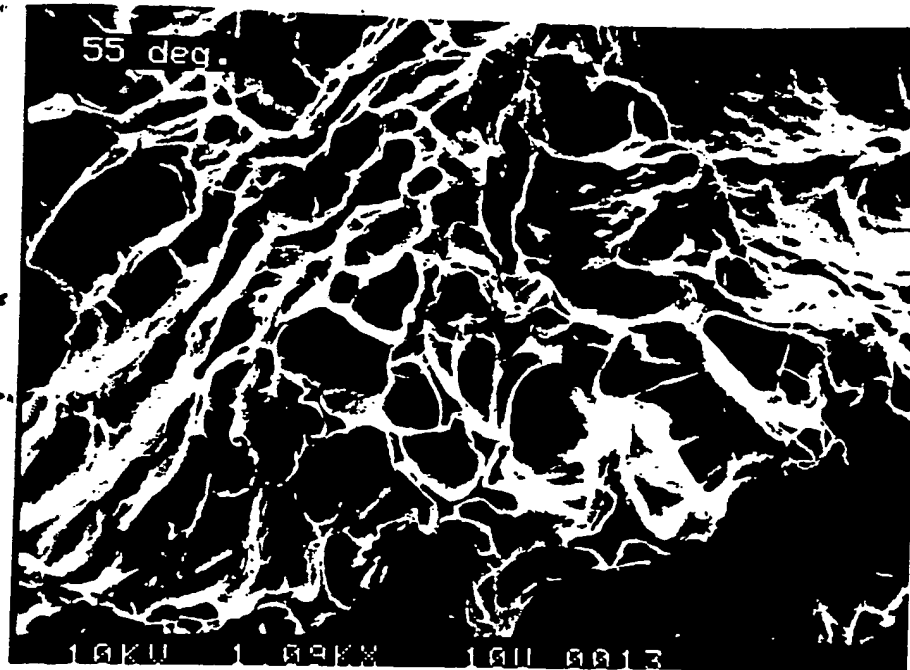
**FIG. 7C**



**FIG. 7D**  SUBSTITUTE SHEET

FIG. 8

ALKALINE PHOSPHATASE (u/mg protein)



OP(5μg)/SOLVENT(μl)

FIG. 9A

CALCIUM CONTENT (μg/mg tissue)



OP (5μg)/SOLVENT (μl)
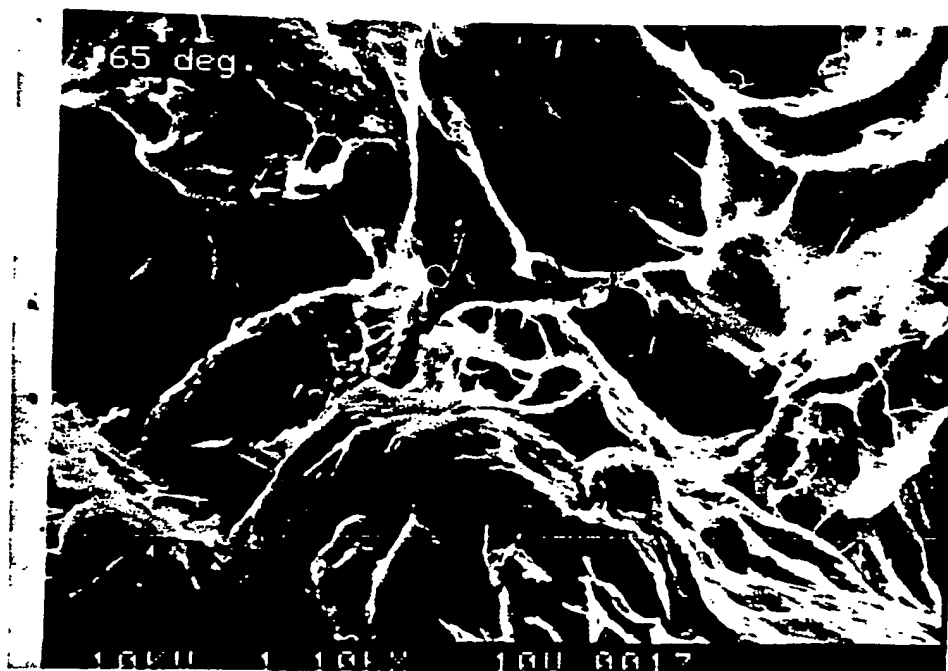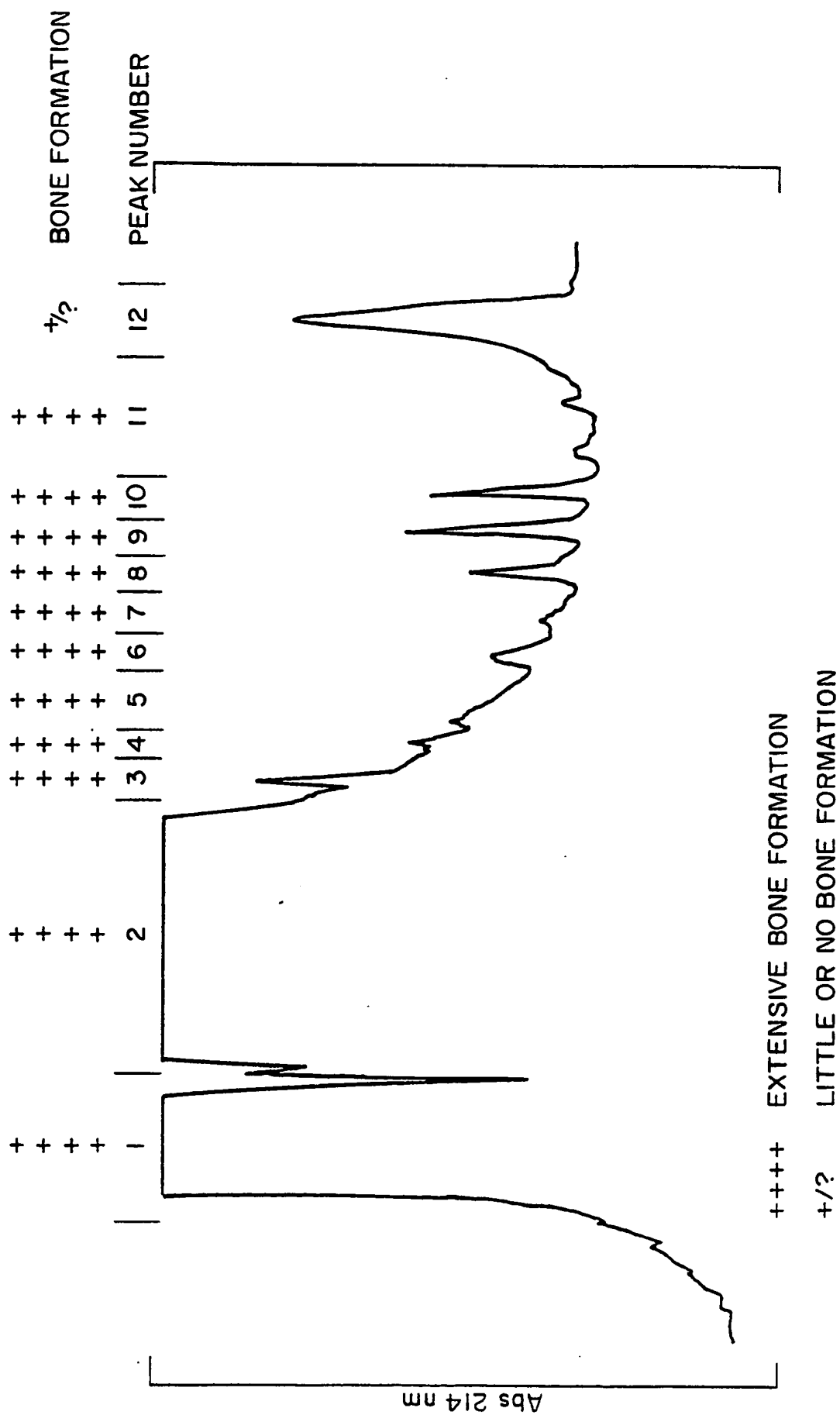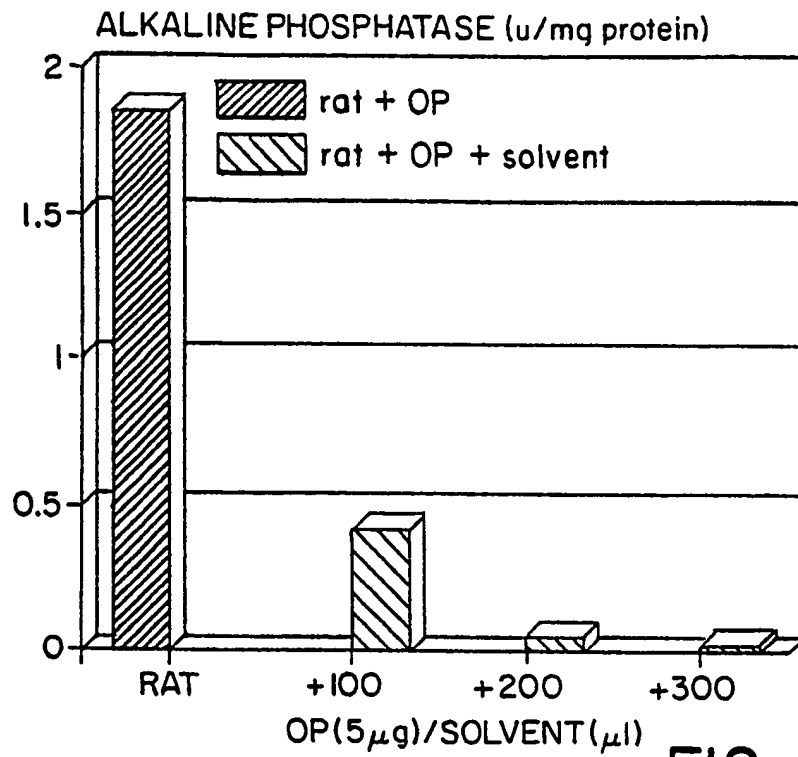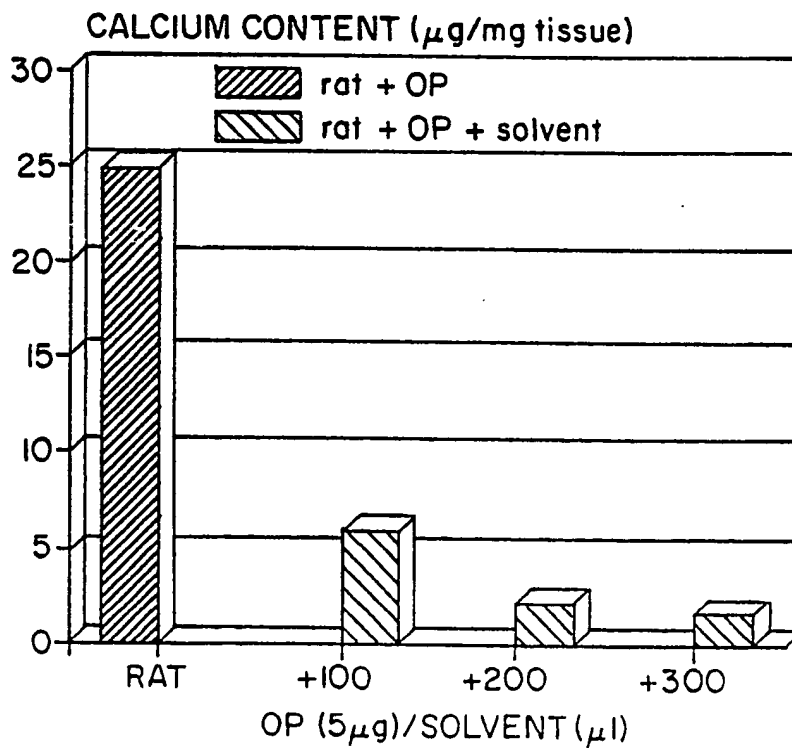
FIG. 9B
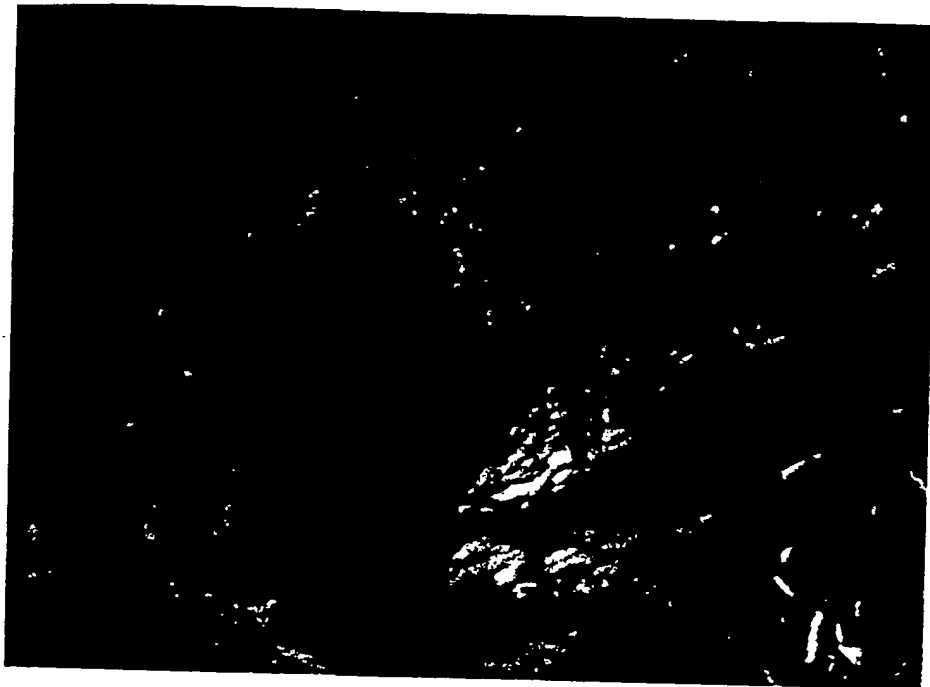
**SUBSTITUTE SHEET**

## FIG. 10A



## FIG. 10B
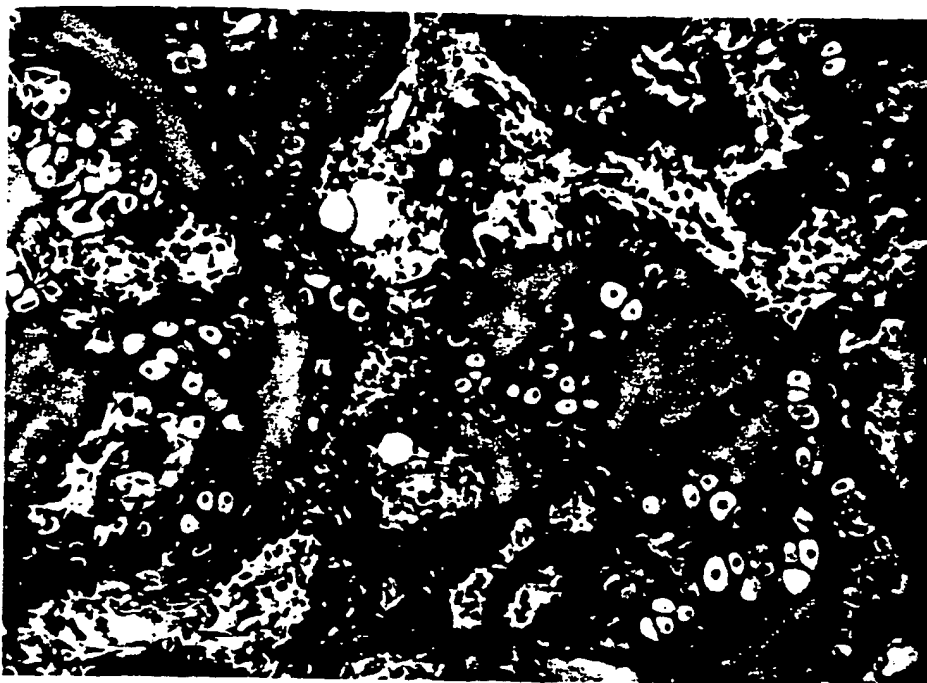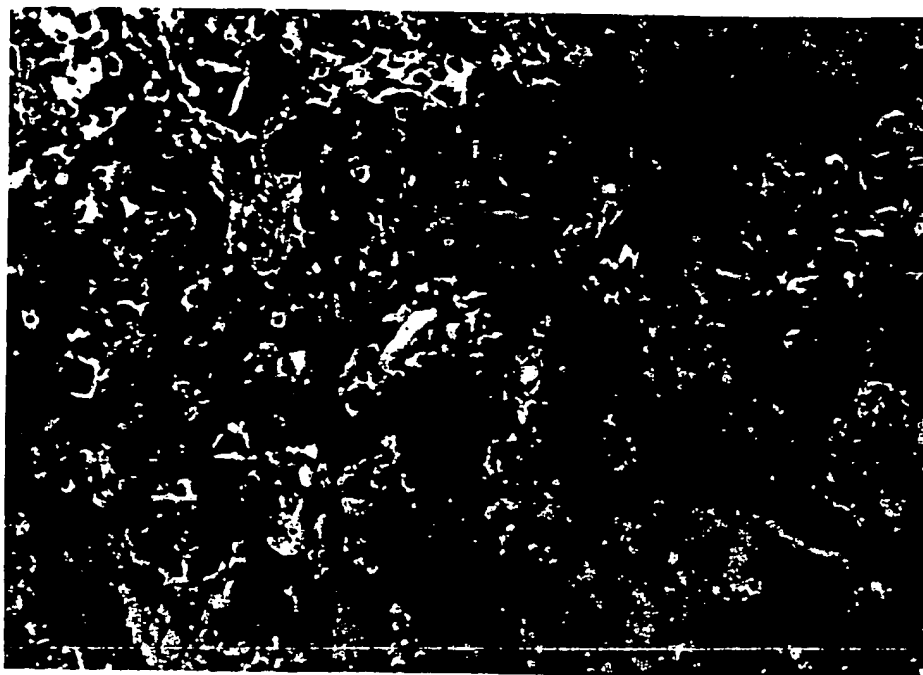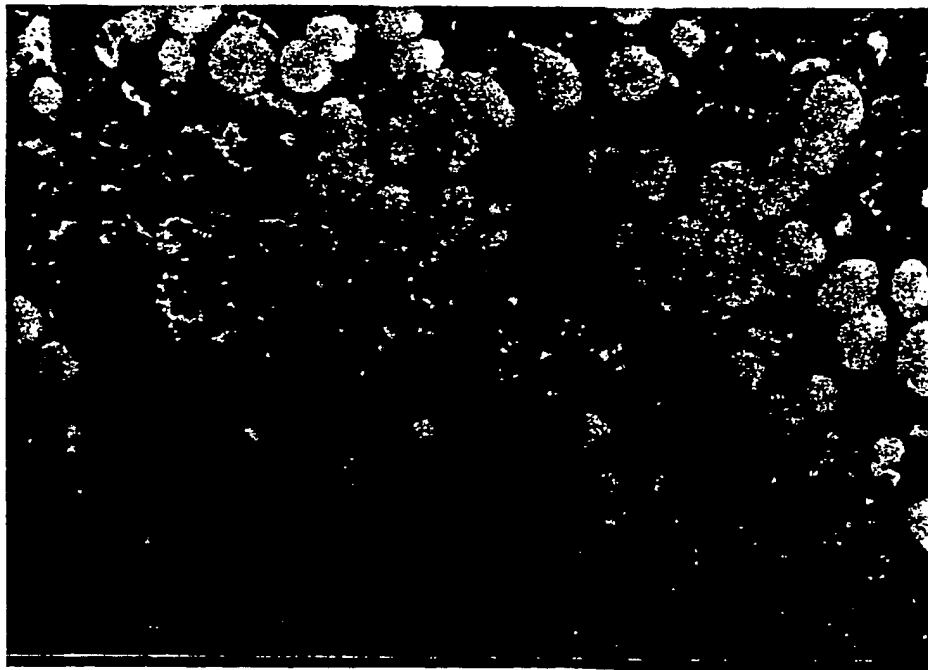
FIG. 10C



FIG. 10D

FIG. 10E



FIG. 10F

FIG. 11

FIG. 12

20/20

ALKALINE PHOSPHATASE (u/mg protein)



FIG. 13A

CALCIUM CONTENT (μg/mg tissue)



FIG. 13B

SUBSTITUTE SHEET
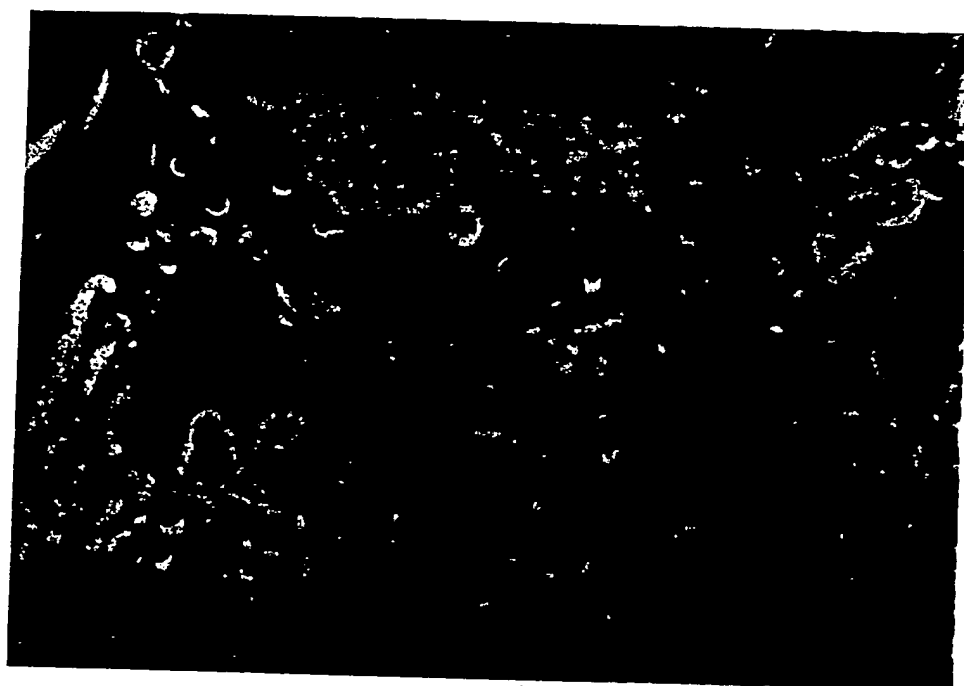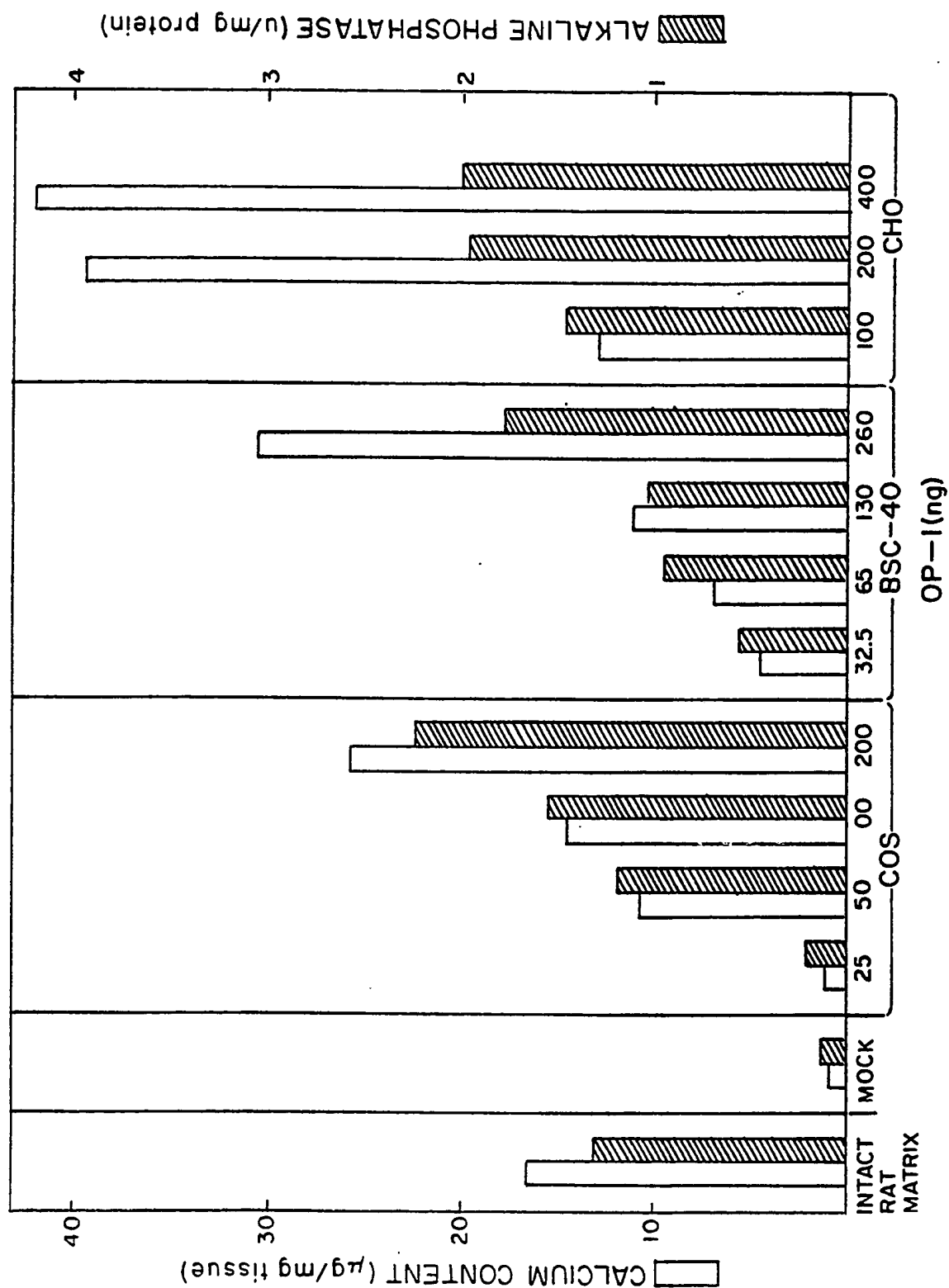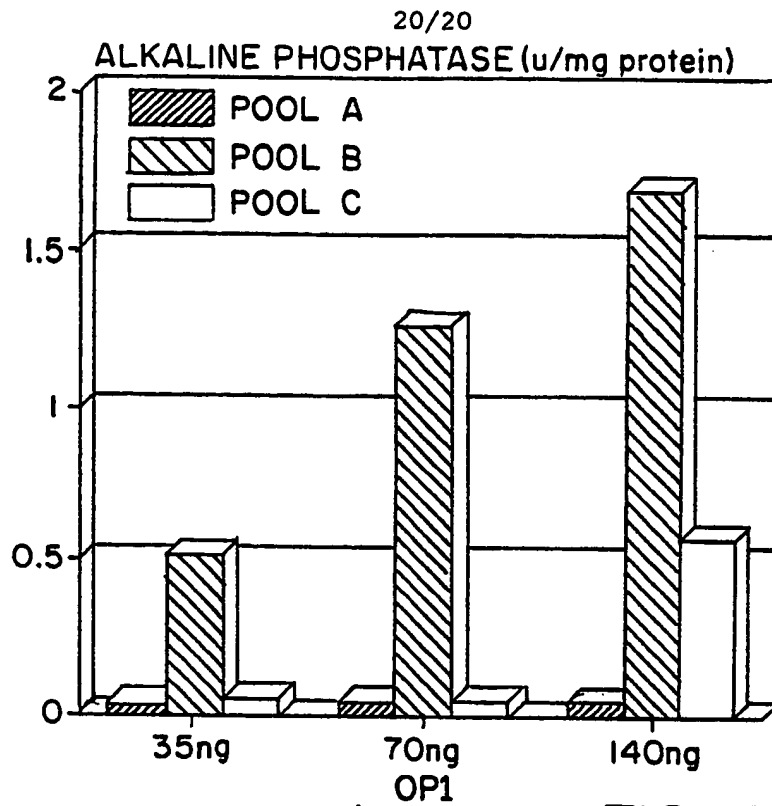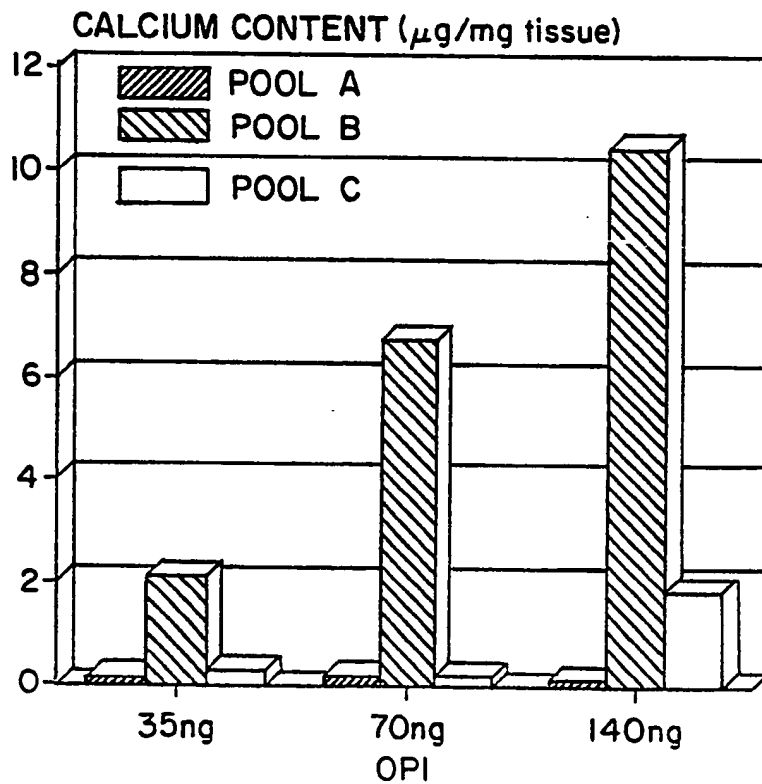
# INTERNATIONAL SEARCH REPORT

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): C07K 15/00,15/06,15/14,17/02; C09H 1/02; A61K 37/12
U.S. CL. 530/350,356,840; 514/2,21; 424/423,424,426; 128/DIG 8

## II. FIELDS SEARCHED

| Minimum Documentation Searched 7 | |
|---|---|
| Classification System | Classification Symbols |
| U.S. | 530/350, 356, 840; 514/2, 21; 424/423, 424, 426 128/DIG 8 |

| Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8 |
|---|
| |

## III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

| Category * | Citation of Document, 11 with indication, where appropriate, of the relevant passages 12 | Relevant to Claim No. 13 |
|---|---|---|
| Y | US, A, 4,394,370 (JEFFERIES) 19 JULY 1983 See column 3, lines 10-25 for the demineral- ized bone powder and Example 1 for prepara- tion of the matrix. | 1-22 & 24 |
| Y | EP, A, 0 148 155 (SEN) 10 JULY 1985 See page 18 for production of mineralized bone powder. | 1-22 & 24 |
| Y | EP, A, 0.182 483 (NATHAN) 28 MAY 1986 See page 9, line 31 to page 10, line 23 for production of demineralized bone powder. | 1-22 & 24 |
| Y | WO, A, WO88/00205 (WANG) 14 JANUARY 1988 See page 11, lines 2-19 for production of a matrix and page 12, line 12 to page 13, line 27 for production of matrix material. | 1-24 |
| Y | Proc. Natl. Acad. Sci., USA, Vol. 80, November 1983, (SAMPATH), "Homology of bone- inductive proteins from human, monkey, bovine and rat extracellular matrix" pages 6591-6595. See "Materials and Methods" for production of the matrix. | 1-22 & 24 |

* Special categories of cited documents: 10
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such docu- ments, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 13 DECEMBER 1990 | 05 FEB 1991 |
| International Searching Authority | Signature of Authorized Officer |
| ISA/US | Nathan M. Nutter |

Form PCT/ISA/210 (second sheet) (Rev.11-87)

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
|---|---|---|
| Y | Proc. Natl. Acad. Sci., Vol. 78, No. 12, USA, December 1981 (SAMPATH), "Dissociative extraction and reconstitution of extracellular matrix components involved in local bone differentiation". Pages 7599-7603. See page 7599 "Preparation of Demineralized Bone", See entire section. | 1-22 & 24 |
| A | US, A, 4,172,128 (THIELE) 23 OCTOBER 1979 | 1-24 |
| A | US, A, 4,294,753 (URIST) 13 OCTOBER 1981 | 1-24 |
| A | US, A, 4,434,094 (SEYEDIN) 28 FEBRUARY 1984 | 1-24 |
| A | US, A, 4,563,350 (NATHAN) 07 JANUARY 1986 | 1-24 |
| A | US, A, 4,563,489 (URIST) 07 JANUARY 1986 | 1-24 |
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| A | US, A, 4,789,663 (WALLACE) 06 DECEMBER 1988 | 1-24 |
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| A,P | US, A, 4,894,441 (MENICAGLI) 16 JANUARY 1990 | 1-24 |
| A | WO, A, WO86/00526 (CAPLAN) 30 JANUARY 1986 | 1-24 |
| A,P | WO, A, WO89/09605 (BENTZ) 19 OCTOBER 1989 | 23 |
| A,P | WO, A, WO89/10409 (WANG) 02 NOVEMBER 1989 | 1-24 |
| A | EP, A, 0 128 041 (BAYLINK) 12 DECEMBER 1984 | 23 |
| A | EP, A, 0 169 001 (SABELMAN) 22 JANUARY 1986 | 1-22 & 24 |
| A | EP, A, 0 169 016 (SEYEDIN) 22 JANUARY 1986 | 23 |
| A | EP, A, 0 212 474 (URIST) 04 MARCH 1987 | 1-24 |
| A | EP, A, 0 230 647 (FUJIOKA) 05 AUGUST 1987 | 1-24 |
| A | EP, A, 0 309 241 (CHU) 29 MARCH 1989 | 1-24 |
| A | Collagen Research, Vol. 1/1981 A.H. Reddi "Cell Biology and Biochemistry of Endochondral Bone Development" pages 209-226. | 1-24 |

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) | | |
|---|---|---|
| Category * | Citation of Document, [16] with indication, where appropriate. of the relevant passages [17] | Relevant to Claim No [18] |
| A | The Lancet, May 2, 1981, Julie Glowacki "Application of the Biological Principle of Induced Osteogenesis for Craniofacial Defects" pages 959-963. | 1-24 |
| A | Journal of Biomedical Materials Research, Vol. 19, 1985, A.H. Reddi "Implant-stimulated interface reactions during collageous bone matrix-induced bone formation" pages 233-239. | 1-24 |
| A | Clinical Orthopaedics and Related Research Number 187, July/August 1984, Marshall R. Urist "$\beta$-tricalcium Phosphate Delivery System for Bone Morphogenetic Protein" pages 277-280. | 1-24 |
| A | Biotechnology and Bioengineering. Vol. XXVI, 1984 Janie M. strand "A Modified Matrix Perfusion-Micro-carrier Bead Cell Culture System. I Adaption of the Matrix Perfusion System for Growth of Human Foreskin Fibroblasts" pages 503-507. | 1-24 |
| A | Clinical Orthopaedics and Related Research, Number 232, July 1988 Stephan D. Cook "Hydroxyapatite-Coated Titanium for Orthopaedic Implant Applications" pages 225-243. | 1-24 |
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| A | Int. J. Orla Maxillofac. Surg. Vol. 17, 1988 J.R. Deatherage "A review of matrix-induced osteogenesis with special reference to its potential use in craniofacial surgery" pages 395-399. | 1-24 |
| A | J. Bone JointSurg. Vol. 70-B, 1988 Per Aspenberg "Failure of Bone Induction by Bone Matrix in Adult Monkeys" pages 625-627. | 1-24 |